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13. ABSTRACT *(Maximum 200 Words)*

Glutamic acid can kill nerve cells during stroke, ischemia, trauma and perhaps in neurodegenerative disease such as Parkinson's. Glutamate kills cells through ionotropic receptor mediated excitotoxicity or via an oxidative stress pathway called oxidative glutamate toxicity. Oxidative stress is also involved in the toxicity of some chemical warfare agents. A major goal of this proposal has been to identify reagents and conditions which protect nerve cells from neurotoxins and oxidative stress, and to determine when in the programmed cell death pathway the neuroprotective agent acts. Toward this end it was shown during the last year that the activation of dopamine D4 receptors protects nerve cells by blocking the activation of cGMP activated calcium channels which regulate calcium influx. Flavonoids, a group of compounds found in plants, were shown for the first time to protect cells by three distinct mechanisms depending upon their structure. These include inhibiting cGMP gated calcium influx, preventing the loss of the intracellular antioxidant glutathione, and by acting as an antioxidant *per se*. Finally, preliminary experiments were carried out to determine if agents which protect nerve cells from glutamate toxicity also protect from arsenite, cisplatin, and hydrogen peroxide toxicity.

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INTRODUCTION

One of the oxidative stress toxicity paradigms which we study is a form of programmed cell death called oxidative glutamate toxicity. In most nerve cells, glutamate can cause the inhibition of cystine uptake through the cystine/glutamate antiporter, resulting in the depletion of the essential amino acid cysteine (reduced cystine). This leads to the near complete loss of glutathione (GSH), new protein synthesis, the activation of lipoxygenase, peroxide production, guanylate cyclase activation, and finally the opening of cGMP gated calcium channels. We have been seeking pharmacological reagents which block this pathway with the hope that they will be effective against other forms of CNS cell death. Dopamine contributes to several CNS pathologies, including schizophrenia and Parkinson's disease, and high does of dopamine, as with glutamate are toxic, acting as pro-oxidants. In contrast to most of the previous work which used high doses of dopamine to generate a neurotoxic effect, we showed that low (1μM) concentrations of dopamine and related agonists are neuroprotective.

BODY

Dopamine and D4 Receptors

The protective effects of dopamine, apomorphine and apocodeine, but not epinephrine and norepinephrine, are antagonized by dopamine D4 antagonists (Ishige et al., 2001, manuscript enclosed). A dopamine D4 agonist also protects and this protective effect is inhibited by U101958, a dopamine D4 antagonist. Although the protective effects of some of the catecholamines are correlated with their antioxidant activities, there is no correlation between the protective and antioxidant activities of several other ligands. Normally glutamate causes an increase in reactive oxygen species (ROS) and intracellular Ca^{2+}. Apomorphine partially inhibits glutamate-induced ROS production and blocks the opening of cGMP-operated Ca^{2+} channels which lead to Ca^{2+} elevation in the late part of the cell death pathway. These data suggest that the protective effects of apomorphine on oxidative stress-induced cell death are mediated by dopamine D4 receptors via the regulation of cGMP-operated Ca^{2+} channels.

Flavonoids

Flavonoids are a group of several hundred diphenylpropanes which are widely distributed in plants and are generally thought to be beneficial dietary supplements, perhaps working as antioxidants. Since we have previously shown that several aromatic antioxidants are able to protect nerve cells from oxidative stress induced toxicity. The goal of this research is to identify potent neuroprotective molecules which may have clinical use. Because flavonoids are relatively non-toxic natural products, they may be of use in this regard. Toward this end we screened a large number of flavonoids for their protective effect against glutamate-induced toxicity and five other forms of oxidative stress to HT22 hippocampal neurons as well as primary rat cortical neurons. In addition, it was asked where in the glutamate-induced cell death program the individual flavonoid acts. Many but not all of the tested flavonoids protect cells from the various forms of oxidative stress (Ishige et al., manuscript enclosed). Three structural requirements of flavonoids for protection are the hydroxylated C3, an unsaturated C ring, and hydrophobicity. We also found three distinct mechanisms of protection. These include increasing intracellular GSH, directly lowering levels of ROS, and preventing the influx of Ca^{2+} despite high levels of ROS. In addition, individual flavonoids can protect by more than one mechanism. For example quercetin and fisetin alter GSH metabolism and act as antioxidants at the same time. Baicalein and luteolin can act as lipoxygenase inhibitors but they can also act as antioxidants. Finally, some flavonoids may protect the cells from glutamate by directly inhibiting ROS production by mitochondria, as well as being antioxidants. These data show that the mechanism of protection from oxidative insults by flavonoids is highly specific for each compound. Since cellular oxidative stress is an important factor in various diseases, including arteriosclerosis, ischemia, trauma, Alzheimer's disease, Parkinson's disease, and AIDS as well as aging itself, flavonoids may have multiple beneficial effects in the treatment of these conditions.

Commonality of Cell Death Programs

A goal of this proposal is to understand the mechanisms involved in nerve cell death in response to toxins and to determine to what extent the toxic response pathways are shared between

toxins. Perhaps more importantly it is necessary to determine if there is any overlap with respect to the protective resistance mechanisms used by nerve cells to protect themselves from one toxin or another. To this end, two sets of experiments were done. In the first, a clonal cell line was selected which is very resistant to glutamate. It was then asked if this cell line is more or less resistant to other toxins. In the second set of experiments, we challenged the HT22 cell line with a variety of toxins, and then determined if reagents known to protect cells from glutamate also protected against the other toxins.

By growing HT22 hippocampal nerve cells in high concentrations of glutamate for extended periods of time it is possible to select for clones which are resistant to glutamate (Sagara et al. 1998). One of these glutamate resistant clones, HT22r2, was used to determine if cells resistant to the form of oxidative stress initiated by glutamate toxicity were also resistant to other forms of toxicity. Table 1 shows that cells resistant to glutamate toxicity were generally resistant to other types of toxic insults. These include BSO, an inhibitor of GSH synthesis which depletes GSH stores and the reduction of exogenous cystine (Δ cys), which also depletes GSH. Glutamate resistant cells are about 5-fold more resistant to glucose starvation, a condition which occurs when cells are starved for blood in conditions of ischemia and stroke. The HT22r2 line is also about 10-fold more resistant to hydrogen peroxide and arsenite. Arsenite is toxic via an ill-defined mechanism, but has been shown to cause increases in ROS production which damage DNA (Wang et al., 2001). Rotenone also is toxic via an increase in ROS production, and is thought to be the cause of some forms of Parkinson's disease and is used to generate a mouse model for the disease (Betarbet et al. 2000). HT22r2 cells are more resistant to rotenone than wild type, but the unusual shape of the dose-response curves makes it difficult to quantitate. Finally, cisplatin causes DNA damage in much the same way as nitrogen mustards. Glutamate resistant cells are not significantly more resistant to cisplatin than their parental cells.

Since a fair amount is known about how HT22r2 cells become resistant to glutamate, some conclusions can be made regarding the common themes which can protect cells from the various toxic insults shown in Table 1. In glutamate resistant cells the expression levels of neither heat shock proteins nor apoptosis-related proteins are changed in the resistant cells (Sagara et al., 1998). In contrast, the antioxidant enzyme catalase, but not glutathione peroxidase nor superoxide dismutase, is more highly expressed in the resistant than in the parental cells. In addition, the resistant cells have enhanced rates of GSH regeneration due to higher activities of the GSH metabolic enzymes γ-glutamylcysteine synthetase and GSH reductase. GSH S-transferases activities are also elevated. As a consequence of these alterations, the glutamate resistant cells are also more resistant to organic hydroperoxides and anticancer drugs that affect these GSH enzymes. It is therefore very likely that GSH plays a central role in protecting cells from multiple forms of toxicity, including arsenite and glucose starvation.

The alternative approach to examining the mechanistic interaction between cell death pathways elicited by different neurotoxins is to treat cells with the different toxins and then ask if reagents which protect cells from glutamate toxicity also protect from arsenite, cisplatin, etc. Table 2 summarizes the results of these preliminary experiments. This data set, although at present incomplete, shows that glutamate, BSO, and hydrogen peroxide toxicity, as well as cystine deprivation are usually rescued by a common set of reagents inhibit oxidative glutamate toxicity (see Maher and Schubert, 2000 for review). Some of these reagents also partially rescue cells from glucose starvation, but most do not alter arsenite and cisplatin toxicity. With the exception of arsenite, these results are in general agreement with those shown in Table 1. The glutamate resistant cells are more resistant to arsenite toxicity, but none of the reagents which block glutamate toxicity clearly block arsenite toxicity. The reason for this is not clear. It can be concluded, however, that regents which block glutamate toxicity can inhibit other forms of cell death caused by pro-oxidant conditions.

KEY RESEARCH ACCOMPLISHMENTS

- The activation of dopamine D4 receptors protects cells from oxidative stress induced cell death.
- Flavonoids and related compounds inhibit neurotoxicity by three distinct mechanisms: enhancing GSH levels, acting as antioxidants, blocking cGMP induced calcium entry.
- Cells selected for resistance to one neurotoxin (glutamate) are resistant to some other neurotoxins.
- Reagents which protect cells from glutamate toxicity also protect cells from peroxide toxicity, but not from arsenite and cisplatin toxicity.

REPORTABLE OUTCOMES

- Two manuscripts (appended)
- Postdoctoral trainee, Yutaka Sagara obtained a job at the University of California, San Diego.
- Third manuscript on shared toxin resistance in preparation

CONCLUSIONS

The data outlined above have identified a series of compounds which protect nerve cells from oxidative stress and related toxic insults and show that resistance to one form of toxicity can lead to resistance to other types of toxicity. Of great interest in terms of Parkinson's disease is the demonstration that dopamine receptor D4 activation leads to neuroprotection. This observation explains a somewhat confused literature which has shown that in some cases catecholamines can protect cells from toxic insults at low concentrations and act as toxins at higher concentrations. It will therefore be of great interest to understand in detail the D4 receptor signaling pathways which lead to neuroprotection.

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Table 1. Glutamate Resistant Lines are Resistant to Other Forms of Toxicity

	HT22	HT22 resistant clone 2
Glutamate *	1.5mM	0. 20mM
BSO *	5μM	0. 200μM
Δ Cys +	50%	8%
Δ Glucose +	10%	2%
H_2O_2 *	20μM	200μM
Arsenite *	6μM	95μM
Cisplatin *	50-100μM	100μM
rotenone ‡		more resistant

* ED_{50}

+ percent of concentration in normal medium

‡ incomplete killing, toxicity curves very flat

Table 2. Protection from Various Toxins

Reagent	Glutamate	BSO	H2O2	Δ cys*	Δ glu*	Arsenite	Cisplatin
YVADcmk [1]	yes	yes	yes	yes	partial	partial	no
Cobalt [2]	yes	yes	yes	yes	partial	no	no
NDGA [3]	yes		yes	yes	no	no	no
DHPG [4]	yes		no	yes	no	no	no
PD 168.007 [5]	yes	no	yes	yes		no	no
Curcumin [6]	yes	yes	yes	yes		no	no
DPI [7]	yes	no	no	yes			
LY83583 [8]	yes	yes		yes			

* Δ cys and Δ glu: cystine and glucose starvation

1. Caspase inhibitor
2. Inhibits Ca^{2+} influx
3. Lipoxygenase inhibitor
4. Group I metabotropic glutamate receptor agonist
5. Dopamine D4 receptor agonist
6. Antioxidant
7. Diphenyleneiodonium: mitochondrial ROS inhibitor
8. Guanylate cyclase inhibitor

The Journal of Neuroscience, August 15, 2001, 21(16):6069–6076

The Activation of Dopamine D4 Receptors Inhibits Oxidative Stress-Induced Nerve Cell Death

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Oxidative stress is thought to be the cause of nerve cell death in many CNS pathologies, including ischemia, trauma, and neurodegenerative disease. Glutamate kills nerve cells that lack ionotropic glutamate receptors via the inhibition of the cystine–glutamate antiporter x_c^-, resulting in the inhibition of cystine uptake, the loss of glutathione, and the initiation of an oxidative stress cell death pathway. A number of catecholamines were found to block this pathway. Specifically, dopamine and related ligands inhibit glutamate-induced cell death in both clonal nerve cell lines and rat cortical neurons. The protective effects of dopamine, apomorphine, and apocodeine, but not epinephrine and norepinephrine, are antagonized by dopamine D4 antagonists. A dopamine D4 agonist also protects, and this protective effect is inhibited by U101958, a dopamine D4 antagonist. Although the protective effects of some of the catecholamines are correlated with their antioxidant activities, there is no correlation between the protective and antioxidant activities of several other ligands. Normally, glutamate causes an increase in reactive oxygen species (ROS) and intracellular Ca^{2+}. Apomorphine partially inhibits glutamate-induced ROS production and blocks the opening of cGMP-operated Ca^{2+} channels that lead to Ca^{2+} elevation in the late part of the cell death pathway. These data suggest that the protective effects of apomorphine on oxidative stress-induced cell death are, at least in part, mediated by dopamine D4 receptors via the regulation of cGMP-operated Ca^{2+} channels.

Key words: HT22 cells; cell death; apomorphine; apocodeine; dopamine D4 receptors; glutamate; cGMP

Dopamine and its five receptor subtypes play diverse roles in the CNS. Their activation is thought to adversely contribute to several neuropathological disorders, including Parkinson's disease and schizophrenia (Seeman and Van Tol, 1994; Sokoloff and Schwartz, 1995). In addition, dopamine may have a neuroprotective role. Catecholamines, such as dopamine, norepinephrine, and epinephrine, are thought to protect nerve cells at low doses by virtue of their antioxidant activities, but are neurotoxic at high doses, acting as pro-oxidants (Noh et al., 1999). It has also been reported that dopamine receptor agonists have neuroprotective effects that are caused by nonreceptor-mediated mechanisms. For example, bromocriptine and apomorphine act as free radical scavengers (Yoshikawa et al., 1994; Sam and Verbeke, 1995; Grünblatt et al., 1999). In addition, dopamine D3 receptors are not critical for the neuroprotection by the D3 agonist, pramipexole, in 3-acetyl pyridine-treated rats (Sethy et al., 1997). In contrast, some recent reports suggest receptor-mediated mechanisms for protection by dopamine receptor agonists. For example, bromocriptine protects dopaminergic neurons from levodopa-induced toxicity by stimulating dopamine D2 receptors (Takashima et al., 1999).

HT22 cells are immortalized mouse hippocampal cells and can be considered a model of oxidative toxicity on exposure to glutamate. HT22 cells have no ionotropic glutamate receptors (Maher and Davis, 1996), but exogenous glutamate blocks cystine uptake into the cells via the inhibition of the glutamate–cystine antiporter, resulting in decreases in intracellular cysteine and glutathione (GSH). GSH is the major intracellular antioxidant, and its loss leads to an inability of the cell to deal with pro-oxidant conditions (oxidative stress). After GSH depletion, there is an accumulation of reactive oxygen species (ROS) and a large Ca^{2+} influx, resulting in a form of programmed cell death that is distinct from apoptosis (Murphy et al., 1989; Tan et al., 1998a,b; Maher and Schubert, 2000). The accumulation of intracellular Ca^{2+} is by Ca^{2+} influx through cGMP-operated Ca^{2+} channels (Li et al., 1997b). In this study, we examined the neuroprotective mechanisms of dopamine and related compounds on oxidative stress-induced nerve cell death in HT22 cells and primary rat cortical neurons. It is shown that the activation of D4 receptors is responsible for protection from oxidative stress by dopamine and its analogs.

MATERIALS AND METHODS

Materials. The oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). The chemicals used were: [^{3}H]spiperone (specific activity 610.5 GBq/mmol; NEN, Boston, MA); dopamine receptor D4 affinity purified polyclonal antibody (Chemicon, Temecula, CA); 2′,7′-dichlorofluorescein diacetate (DCF), indo-acetoxymethylester (Indo-1), pluronic F-127, and propidium iodode (all from Molecular Probes, Eugene, OR); haloperidol and L745870 (Tocris Cookson, Ballwin, MO); and 8-(4-clorophenylthio) cGMP (pCPT-cGMP), apomorphine, apocodeine, PD168077, U101958, dopamine, and spiperone (Sigma, St. Louis, MO).

Cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The HT22 cells were propagated in DMEM that was supplemented with 10% fetal bovine serum (FBS). Cell survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is taken up by recycling vesicles in which it is reduced and cycled to the extracellular space (Liu et al., 1997). In the HT22 system, it is a valid measure of cell death when compared with trypan blue-based visual counting and colony formation (Maher and

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Davis, 1996). Briefly, HT22 cells were dissociated with pancreatin (Life Technologies, Gaithersburg, MD) and seeded onto 96-well microtiter plates at a density of 2×10^3 cells per well in 100 μl of the same medium. The next day, cells were treated with various reagents according to the experimental design. Twenty hours after the addition of glutamate, the culture medium was replaced with fresh medium because some of the catecholamines directly reduced MTT at the higher concentrations tested. In all cases, parallel dishes containing no cells were used, and for each drug concentration, cells with drug alone (no glutamate) were used to determine whether the drug had a direct interaction with the cell. These controls ensured that no direct reduction of MTT by the catecholamines occurred, and visual counts were done to confirm the MTT data. In some cases, the calcein AM viability assay (Molecular Probes) was used. For the MTT assay, 10 μl of 2.5 mg/ml MTT solution was added then and incubated at 37°C for 4 hr, and 100 μl of solubilization solution (50% dimethylfomamide, 20% SDS, pH 4.8) was added. The next day, the absorption values at 570 nm were measured (Liu et al., 1997). The results are shown as the percentage of the controls specified in each experiment. The primary cortical cells were prepared as described by Sagara and Schubert (1998). In all cases, cell death was confirmed by visual inspection.

Total antioxidant activity assay. Total antioxidant activity was measured using the procedure described by Miller et al., (1993) and expressed as Trolox, a water-soluble vitamin E analog, equivalent antioxidant capacity (TEAC). The TEAC value is the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of sample under investigation. Briefly, 1 ml of reaction mixture including 2.5 μM metmyoglobin, 150 μM 2,2′-azinobis(3-ethylbenzoline 6-sulfonate), 75 μM H_2O_2, and 0.84% sample or Trolox (for standard) in PBS was incubated for 7.5 min at 30°C; then the absorbance at 734 nM was read at 7.5 min. The data are normalized to 1 mM Trolox (TEAC activity).

GSH, ROS, and Ca^{2+} measurements. Total GSH was measured as described by Tan et al. (1998a), using pure reduced glutathione as the standard. ROS and Ca^{2+} measurements were performed as described by Tan et al. (1998a) by flow cytometry. ROS production and intracellular Ca^{2+} were detected using DCF and Indo-1, respectively. Briefly, the cells were incubated with Indo-1 and pluronic F-127 for 25 min at 37°C; then DCF and pancreatin were added, and cells were incubated for 5 min. Cells were collected and washed once in HEPES buffer supplemented with 2% dialyzed fetal bovine serum. Washed cells were resuspended in HEPES buffer and kept on ice until flow cytometric analysis. DCF data were collected with the 475 nm excitation and 525 nm emission wavelengths and plotted as histograms using the data analysis program CELLQuest (Becton Dickinson, Mountain View, CA). Indo-1 data were collected with two emission wavelengths, 410 nm (FL32) and 485 nm (FL4). FL32 and FL4 reflect the fluorescence of Indo-1 with and without bound Ca^{2+}, respectively. The Ca^{2+} concentration is presented as the ratio of FL32/FL4 (Sagara, 1998; Tan et al., 1998a). Data were analyzed from 10,000 live cells as determined by the lack of propidium iodide fluorescence.

Reverse transcription polymerase chain reaction. Total RNA was prepared from HT22 cells and various tissues of mice and then treated with DNase for 30 min at 15°C. Oligonucleotide primers used for the PCR amplification were: D4–1, 5′-CCTTACCCAGCCTCCGGACGA-3′, which corresponds to nucleotides 764–784 of the mouse D4 receptor sequence; and D4–2, 5′-GACACGAAGCAAGCCGGACA-3′, which is complementary to nucleotides 1018–1037 of the same sequence. PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide.

Western blotting. HT22 cells were collected by scraping in sample buffer. Mouse tissues were homogenized with a Polytron (Kinematica, Basel, Switzerland) for 20 sec in PBS supplemented with a mixture of protease inhibitors (Complete; Roche Molecular Biochemicals, Indianapolis, IN), and then centrifuged at 48,000 × *g* for 30 min at 4°C. The resulting pellets were resuspended in sample buffer (3% SDS, 1% glycerol, 0.5% 2-mercaptoethanol, 0.05% bromophenol blue, and 80 mM Tris-HCl buffer, pH 6.8, with Complete protease inhibitors). The samples were heated for 3 min in boiling water, fractionated on 12% polyacrylamide gels, and electroblotted onto membranes. Dopamine receptor D4 affinity purified polyclonal antibody was used as the primary antibody. Immunoreactive bands were detected with the ECL (Amersham Pharmacia Biotech, Arlington Heights, IL) Western blotting detection reagents.

Binding assays. HT22 cells were homogenized with a Polytron for 20 sec in 15 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, and then centrifuged at 48,000 × *g* for 30 min at 4°C. The resulting pellet, which constitutes the membrane fraction, was resuspended in 50 mM Tris-HCl buffer, pH 7.4. Binding assays were performed as described by Maroto et al. (1995), with minor modifications. Briefly, 0.5 nM [^{3}H]spiperone and the membrane fraction (100–150 μg protein–assay tube) were incubated in 50 mM Tris-HCl buffer, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% ascorbic acid for 2 hr at 4°C. The binding reaction was terminated by rapid filtration through Whatman GF/C filters presoaked in 0.3% polyethylenimine. The filters were immediately washed three times with 4 ml of ice-cold 50 mM Tris-HCl buffer, and radioactivity was measured by liquid scintillation counting. The nonspecific binding was determined in the presence of 100 μM haloperidol. Specific binding was estimated by subtracting nonspecific binding from total binding. Binding assays were done in triplicate. Protein concentration was measured by the Bradford method using bovine serum albumin as the standard.

Statistical analysis. The significance of differences between two groups was assessed by Student's *t* test. The significance of three or more groups was assessed by the Bonferroni test.

RESULTS

Effect of dopamine and related compounds on glutamate-induced cell death

Because there have been suggestions in the literature that catecholamines can protect nerve cells from oxidative stress (Noh et al., 1999; Grünblatt et al., 1999), it was asked whether this phenomenon could be reproduced in a well characterized form of programmed cell death that is initiated by oxidative stress. HT22 cells, which lack ionotropic glutamate receptors, were exposed to increasing concentrations of several catecholamines followed by 2.5 mM glutamate. Cell viability assays performed 20 hr later showed that glutamate alone kills >90% of the cells, whereas dopamine protects HT22 cells in a concentration-dependent manner (Fig. 1*A*). Apomorphine and apocodeine, two other dopamine receptor ligands, are also protective (Fig. 1*A*), whereas epinephrine and norepinephrine are less active. Of the compounds tested, apomorphine was the most effective, followed by apocodeine and dopamine. These compounds did not affect control cell survival, and they also protected cells from higher concentrations of glutamate (data not shown). In addition, apomorphine protects rat cortical neurons lacking ionotropic glutamate receptors from oxidative glutamate toxicity (Fig. 1*B*).

To confirm the data with the MTT assay and to visually present the dramatic effects of the dopamine analogs on rescuing the cells from glutamate toxicity, some of the conditions in Figure 1 were repeated with the cell viability stain calcein AM. Calcein AM is a fluorogenic esterase substrate that passes through the cell membrane and is hydrolyzed inside the viable cell to the green fluorescent product calcein (Vaughan et al., 1995). Then, live cells were quantitated (Fig. 2*A*); photomicrographs of the cells are presented in Figure 2*B*. It is clear that essentially all of the cells were killed by glutamate and that both apocodeine and apomorphine rescue the cells. Quantitation using calcein AM was, within experimental variation, the same as that using the MTT assay (Fig. 1).

Because apomorphine and apocodeine are ligands for dopamine receptors (Van Tol et al., 1991; Seeman and Van Tol, 1994), it is possible that the activation of a dopamine receptor leads to protection. To define the receptor subtype, dopamine receptor antagonists were assayed for their reversal of apomorphine protection in HT22 cells. The protective effects of apomorphine, apocodeine, and dopamine were all inhibited by the D4 antagonists, L745870 and U101958, whereas the weaker protective effects of epinephrine or norepinephrine were not affected by these

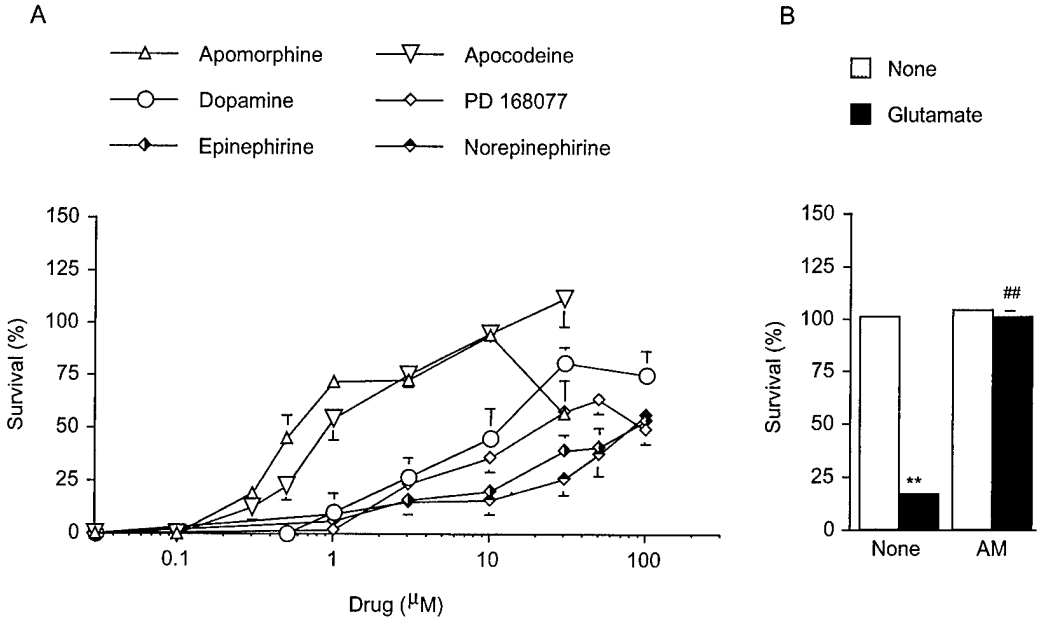

Figure 1. Protective effects of apomorphine and related compounds on glutamate-induced cell death in HT22 cells and primary cortical cells. HT22 (*A*) and 1-d-old primary cortical (*B*) cells were incubated with various drugs and 2.5 mM or 5 mM glutamate, respectively, for 20 hr; then cell survival was measured by the MTT assay. The results are presented as the mean ± SEM relative percentage survival for three or four independent experiments each done in triplicate. *AM*, Apomorphine (1 μM). $^{**}p < 0.01$ (vs no glutamate); $\#\#p < 0.01$ (vs glutamate alone).

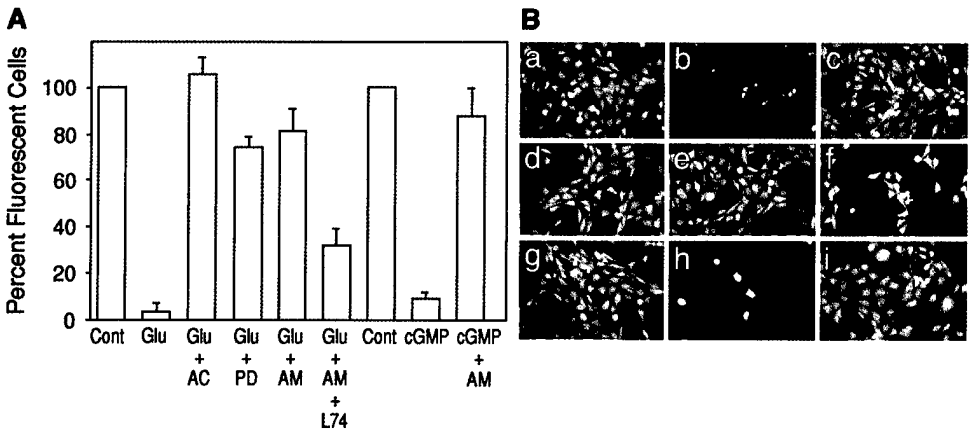

Figure 2. Viability assays using calcein AM. Cells were treated as described below, and viability was determined 20 hr later using calcein AM instead of MTT. *A*, Quantitation of viable cells. Twenty microscopic fields containing between 0 and 80 (control) cells each were scored for viable calcein-positive cells, and the average cells per field in the control (untreated) cultures was given as 100%. The data are presented as the mean cell number relative to control ± SEM. *Cont.*, Control; *Glu*, 2.5 mM glutamate; *AC*, 10 μM apocodeine; *PD*, 50 μM PD168077; *AM*, 1 μM apomorphine; *L74*, 3 μM L745870; *cGMP*, 2.5 mM pCPT-cGMP. The last three columns were from a separate experiment than the first six. *B*, Photomicrographs of calcein-stained HT22 cell cultures after treatment with the reagents described in *A* and Figure 1. The conditions are those described in *A*. *a*, Control; *b*, glutamate; *c*, glutamate plus apocodeine; *d*, glutamate plus PD168077; *e*, glutamate plus apomorphine; *f*, glutamate plus apomorphine plus L745870; *g*, control; *h*, pCPT-cGMP; *i*, pCPT-cGMP plus apomorphine.

antagonists (Fig. 3*A*). The inhibitory effects of dopamine D4 antagonists were not complete, but we could not use higher concentrations because they were toxic. PD168077, a dopamine D4 receptor agonist, also had a significant protective effect on glutamate-induced cell death, and this protective effect was also antagonized by U101958 (Figs. 1, 3*B*). The protective effect of the D4 receptor agonist PD168077 and the antagonistic effect of L745870 are also shown in Figure 2 using the calcein AM viability stain. SCH23390, a dopamine D1 receptor antagonist; sulupiride, a dopamine D2 receptor antagonist; and GR103691, a dopamine D3 antagonist, did not antagonize the protective effects of apomorphine or apocodeine, nor did they affect cell survival in the absence of glutamate (data not shown). These data suggest that the dopamine D4 receptor is involved in the protection of cells from oxidative stress by apomorphine.

Antioxidant activity (TEAC)

It is possible that at least a part of the neuroprotection by apomorphine and related compounds is attributable to their antioxidant activity (Yoshikawa et al., 1994; Grünblatt et al., 1999). To test the antioxidant activities of these compounds, we measured their TEAC values, an index of antioxidant activity *in vitro* (Table 1). Dopamine had the highest antioxidant activity of the compounds shown in Figure 1*A*, and the antioxidant activity of apomorphine tended to be higher than that of epinephrine or norepinephrine. Apocodeine is a weak antioxidant, although it protects cells as well as apomorphine (Fig. 1). In addition, the D4 agonist PD168077, which is also protective, had no antioxidant activity. As shown in Figure 4, the potencies of the protective effects were correlated with antioxidant activities in SKF38393, 7-hydroxy-dipropylaminotetralin (7-OH DPAT), catecholamines,

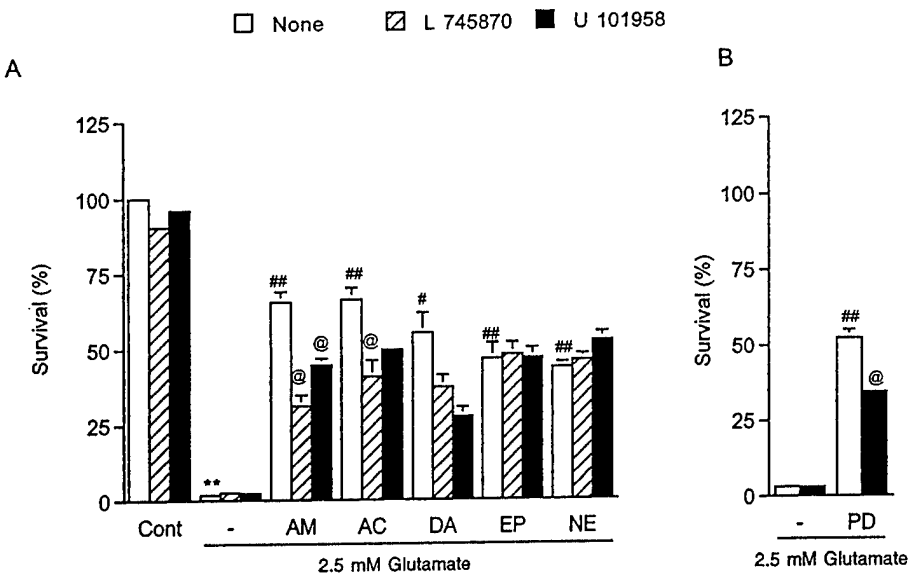

Figure 3. Inhibitory effects of D4 antagonists on the neuroprotective effects by apomorphine and related compounds. *A*, HT22 cells were incubated with glutamate (2.5 mM) and apomorphine (*AM*; 1 μM), apocodeine (*AC*; 1 μM), dopamine (*DA*; 30 μM), epinephrine (*EP*; 100 μM), or norepinephrine (*NE*; 100 μM) in the presence or absence of the D4 antagonists, L745870 (3 μM) or U101958 (10 μM), for 20 hr; then cell survival was measured by the MTT assay. *B*, HT22 cells were incubated with 2.5 mM glutamate and 50 μM PD168077 in the presence or absence of 10 μM U101958 for 20 hr; then cell survival was measured by the MTT assay. The results are presented as the mean ± SEM relative percentage survival for three independent experiments. $^{**}p < 0.01$ (vs control, no glutamate); $\#\#p < 0.01$ (vs glutamate alone); $\#p < 0.05$ (vs glutamate alone); $@p < 0.05$ (vs glutamate in each group). The statistical analysis was performed with the Bonferroni test.

Table 1. TEAC values

Compounds	TEAC (mM)
Apomorphine	1.70 ± 0.07
Apocodeine	0.74 ± 0.05
PD 168077	0.03 ± 0.14
Dopamine	2.40 ± 0.04
SKF 38393	4.11 ± 0.11
7-OH-DPAT	1.34 ± 0.10
Norepinephirine	1.54 ± 0.03
Epinephirine	1.49 ± 0.04
Serotonin	1.86 ± 0.07

TEAC values were measured as described in Materials and Methods. The results are presented as the mean ± SE of four independent experiments.

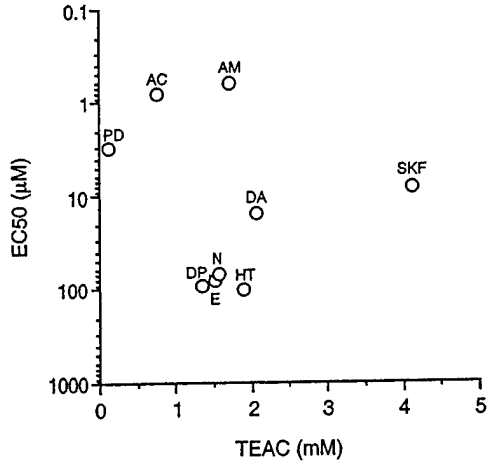

Figure 4. Relationship between the TEAC values and protective effects on glutamate-induced cell death in HT22 cells. TEAC values were obtained from Table 1. EC_{50} values for suppression of 2.5 mM glutamate-induced cell death in HT22 cells were calculated from each concentration–response curve and are presented as the mean of three or four independent experiments. *AM*, Apomorphine; *AC*, apocodeine; *PD*, PD168077; *DA*, dopamine; *SKF*, SKF38393; *DP*, 7-hydroxydipropylaminotetralin (7-OH-DPAT); *N*, norepinephrine; *E*, epinephrine; *HT*, serotonin.

and serotonin. SKF38393 is a D1 agonist and has two phenolic hydroxyl groups. 7-OH DPAT is a D3 agonist and has one phenolic hydroxyl group. In contrast, there is no correlation between antioxidant activity and neuroprotection with apomorphine, apocodeine, or PD168077. It is therefore possible that the first group protects cells by virtue of their antioxidant activity, whereas apomorphine, apocodeine, and PD168077 do so by a different mechanism.

Glutathione, ROS and Ca^{2+} levels

Oxidative glutamate toxicity is associated with the depletion of GSH and the elevation of intracellular ROS and Ca^{2+} (Tan et al., 1998a). To determine where in the programmed cell death pathway apomorphine blocked toxicity, we measured intracellular GSH, ROS, and Ca^{2+} levels. Dopamine, apomorphine, and apocodeine did not prevent glutamate-induced glutathione depletion (Fig. 5*A*), showing that this early event in the oxidative stress pathway is not the target for these compounds. In contrast, apomorphine, apocodeine, and dopamine partially inhibited the ROS elevation (Fig. 5*B*). Dopamine showed the strongest inhibition of ROS production, perhaps because of its powerful antioxidant activity. Finally, all of the drugs inhibited Ca^{2+} elevation completely (Fig. 5*C*). Because the influx of Ca^{2+} is a late event in the oxidative glutamate toxicity death program and because some increase in intracellular Ca^{2+} is required for maximum ROS production (Tan et al., 1998a), it follows that apomorphine and apocodeine may inhibit cell death by blocking the influx of Ca^{2+}.

cGMP induced Ca^{2+} influx

In HT22 cells and cortical neurons, cGMP-dependent Ca^{2+} channels are opened near the end of the glutamate-induced cell death pathway (Li et al., 1997b). To test the possibility that apomorphine and apocodeine modulate these Ca^{2+} channels, we examined the effects of these compounds on cell death that was caused by the cell permeable cGMP analog, pCPT-cGMP. pCPT-cGMP caused cell death in a dose-dependent manner (data not shown), and $CoCl_2$, a nonselective Ca^{2+} channel inhibitor, suppressed pCPT-cGMP-induced cell death (Fig. 6*A*). Apomorphine, apocodeine, PD168077, and dopamine also inhibited pCPT-cGMP-induced cell death (Fig. 6*A*), showing that they act at a site downstream from cGMP in the cell death

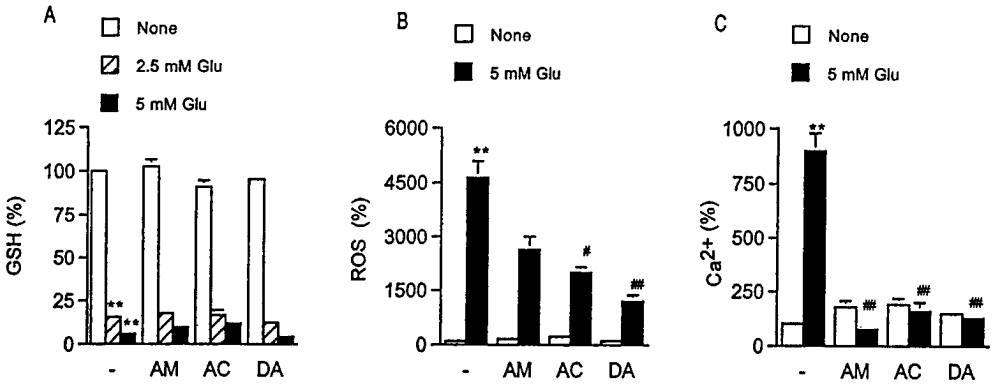

Figure 5. The effects of apomorphine, apocodeine, and dopamine on glutathione (*GSH*) depletion, reactive oxygen species (*ROS*) production, and Ca^{2+} influx by glutamate. Cells were incubated with 2.5 mM (*GSH*) or 5 mM (*GSH*, *ROS*, and *Ca^{2+}*) glutamate and each drug for 8 hr; then GSH, ROS and Ca^{2+} were measured as described in Materials and Methods. GSH was calculated as nanomoles GSH per milligram of protein and presented as a percentage of the control value. ROS and Ca^{2+} were calculated as described in Materials and Methods and presented as a percentage of control. All results are presented as the mean ± SEM for four independent experiments. –, Control; *AM*, apomorphine; *AC*, apocodeine; *DA*, dopamine. $^{**}p < 0.01$ (vs no glutamate); $\#p < 0.05$ and $\#\#p < 0.01$ (vs 5 mM Glu).

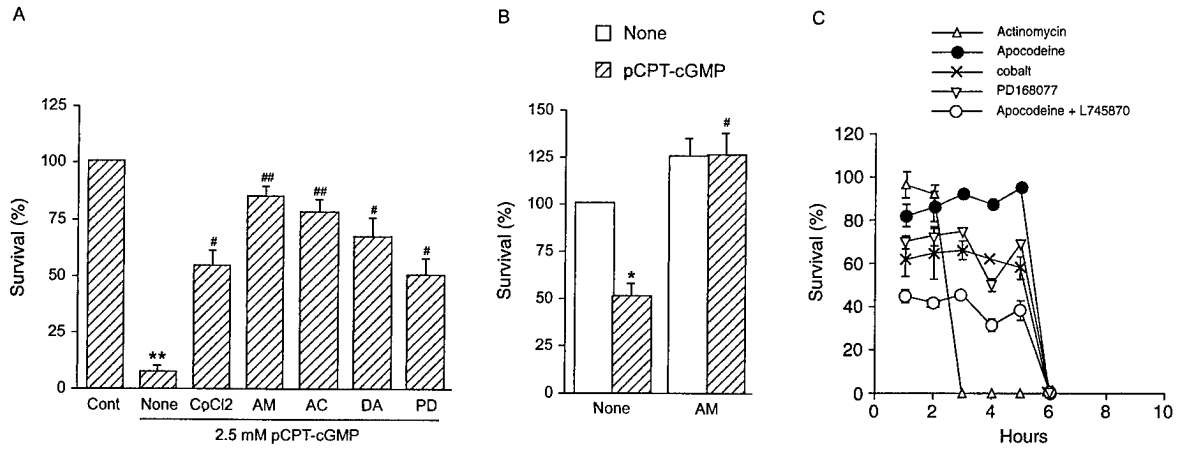

Figure 6. Effects of dopamine receptor ligands on cGMP-induced cell death in HT22 cells and primary cortical neurons. HT22 (*A*) or 1-d-old cultured cortical (*B*) cells were incubated with $CoCl_2$ (50 μM), apomorphine (*AM*; 1 μM), apocodeine (*AC*; 1 μM), dopamine (*DA*; 30 μM), or PD168077 (*PD*; 50 μM), followed by the addition of 2.5 mM pCPT-cGMP. Cell survival was measured by the MTT assay 24 hr later. *C*, Glutamate was added to all samples at 0 time, and 20 μM $CoCl_2$, 1 μM apocodeine, 50 μM PD168077, 1 μM apocodeine plus 3 mM L745870 or 0.1 μg/ml actinomycin D were added at 2 hr intervals up to 10 hr; cell viability was determined after 20 hr. The results are presented as the mean ± SEM relative percentage survival for three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ (vs no glutamate); $\#p < 0.05$ and $\#\#p < 0.01$ (vs 5 mM glutamate).

pathway. The results with apomorphine using calcein AM are shown in Figure 2. In addition, pCPT-cGMP induced cell death in primary cortical neurons, and these cells were protected by apomorphine (Fig. 6*B*).

If dopamine and its analogs protect cells primarily via the inhibition of Ca^{2+} influx, then it would be predicted that they protect cells from glutamate toxicity when added very late in the cell death program in which the Ca^{2+} influx occurs (Li et al., 1997b; Tan et al., 1998a). In addition, this inhibition should parallel the time course for cobalt protection, and both should occur much later than happens with actinomycin D, for the requirement for mRNA synthesis is a very early event (Tan et al., 1998a). To test this possibility, actinomycin D, $CoCl_2$, the D4 agonist PD168077, apocodeine or apocodeine, plus D4 antagonist L745870 were added to HT22 cells at 2 hr intervals after the addition of glutamate, and cell viability was determined 20 hr later. Figure 6*C* shows that cells are protected from glutamate when $CoCl_2$, apocodeine, or the D4 agonist is added up to 6 hr after glutamate, whereas the protective effect of apocodeine was partially reversed by L745870. Actinomycin D no longer inhibited toxicity when added after 2 hr. These results show that apocodeine protects cells late in the cell death pathway via the activation of D4 receptors and are consistent with both the cGMP and Ca^{2+} influx data.

Dopamine receptors in HT22 cells

The above experiments suggest that HT22 cells have dopamine D4 receptors that mediate the protective response. Three sets of experiments were done to determine whether the HT22 cells express the D4 receptor. D4 receptor mRNA was assayed by reverse transcription polymerase chain reaction (RT-PCR), Western blot experiments were performed to examine protein levels, and ligand binding assays were performed to examine receptor function. In RT-PCR, amplification with D4–1 and D4–2 primers generated a 195 bp cDNA fragment from the HT22 cells, mouse hippocampal RNA, and mouse cortical neuron RNA (Fig. 7*A*). The corresponding band was not detected in mouse liver or kidney. Similarly, Western blots with an antibody specific to D4 receptors showed that HT22 cells, mouse hippocampal membranes, and mouse cortical neurons have dopamine D4 re-

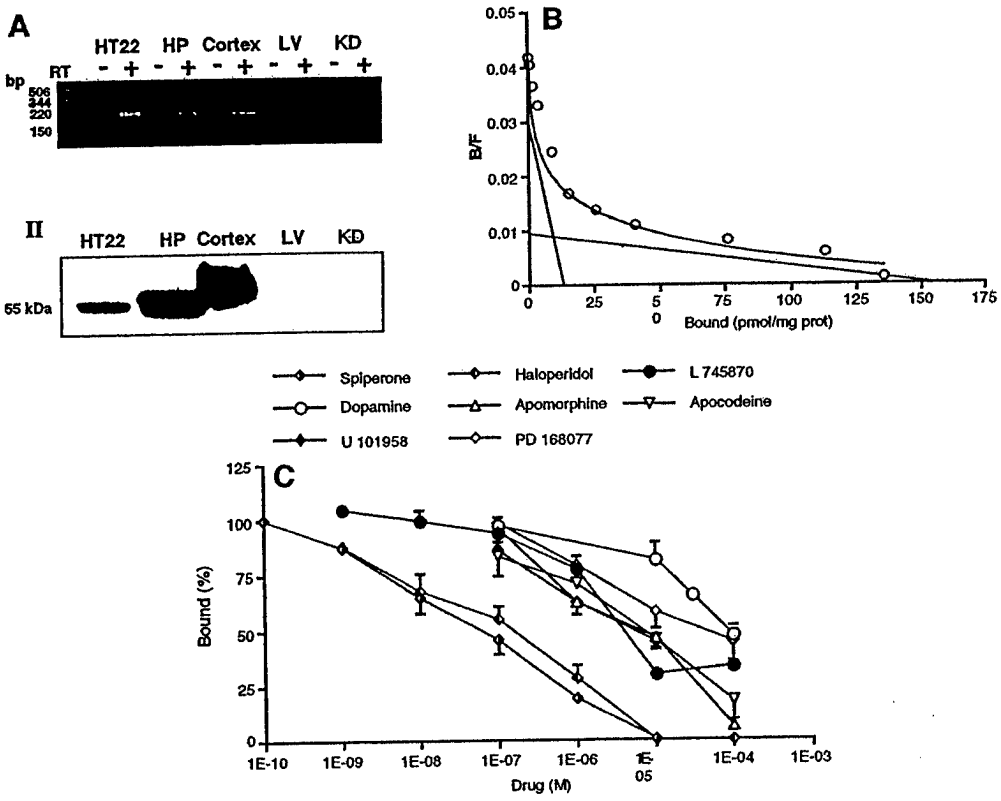

Figure 7. The expression of D4 receptors in HT22 cells. *A*, RT-PCR and Western blot analysis of dopamine D4 receptors in HT22, hippocampal, and cortical cells. DNase-treated total RNA with (+) or without (-) reverse transcription was amplified by PCR. The estimated size of the PCR products was 195 bp. *II*, Lanes of HT22 cell lysates contained 40 μg of protein, and the other lanes contained 20 μg of protein. The estimated molecular weight of the D4 receptor is 55 kDa in HT22 and hippocampal neurons and slightly larger in cortical neurons. *B*, Representative Scatchard plot of [^{3}H]spiperone binding. The binding assay was performed as described in Materials and Methods by using concentrations of spiperone between 0.5 nM and 2.5 μM. *C*, Displacement curves for [^{3}H]spiperone binding. The binding assay was performed as described in Materials and Methods. The results are presented as the mean ± SEM of three to five independent experiments. IC_{50} values are shown in Table 3.

Table 2. K_d and B_{max} values for [^{3}H]spiperone binding

	K_d (nM)	B_{max} (pmol/mg protein)
High-affinity sites	7.21 ± 0.56	28.88 ± 13.08
Low-affinity sites	446.09 ± 177.70	174.02 ± 51.99

Scatchard analysis was performed as shown in Figure 7. The results are presented as the mean ± SE of four independent experiments.

Table 3. IC_{50} values for [^{3}H]spiperone binding

Ligand	IC_{50} (μM)
Spiperone	0.11 ± 0.03
Haloperidol	0.31 ± 0.15
L 745870	3.06 ± 1.21
U 101958	7.66 ± 3.33
PD 168077	26.10 ± 3.12
Apomorphine	5.96 ± 2.15
Apocodeine	6.41 ± 1.49
Dopamine	77.82 ± 10.10

IC_{50} values were calculated from each displacement curve. The results are presented as the mean ± SE of three to five independent experiments.

ceptors (Fig. 7*AII*). In the liver and kidney, this band was not observed. The molecular weight of these proteins was estimated at 55 kDa. These data show that HT22 cells and the tissue from which they were derived, mouse hippocampus, both express D4 receptors.

To further characterize the dopamine D4 receptors in HT22 cells, we performed binding assays. Scatchard plots for the dopamine receptor antagonist [^{3}H]spiperone binding to HT22 cells are curvilinear (Fig. 7*C*), fitting a two site binding model. As shown in Table 2, the K_d values for high- and low-affinity sites are 7.21 ± 0.56 nM and 466 ± 177 nM, respectively, and the B_{max} values are 28.88 ± 13.08 pmol/mg protein and 174.02 ± 51.99 pmol/mg protein, respectively. Displacement experiments showed that various nonselective and D4-selective ligands inhibited [^{3}H]spiperone binding (Fig. 7*C*, Table 3). The most effective displacing agent was spiperone, followed by haloperidol. The IC_{50} value for apomorphine was almost the same as that of apocodeine. The displacing potency of dopamine was the same or weaker than that of the D4 ligands, U101958, L745870, and PD168077.

DISCUSSION

The above data show that the activation of dopamine D4 receptors can play a role in the protection of nerve cells from oxidative stress-induced cell death. This conclusion is based on the following observations. (1) Apomorphine, apocodeine, and dopamine all protect the HT22 mouse hippocampal nerve cell line and rat cortical neurons from oxidative stress induced by oxidative glutamate toxicity (Figs. 1, 2). (2) This protective effect is reversed by D4 antagonists, but not by D1, D2, or D3 antagonists (Fig. 3). (3) A selective D4 agonist, PD168077, also protects neurons from oxidative stress (Figs. 1, 2). (4) The protective effects of the D4 agonists cannot be explained by their inherent antioxidant properties alone (Fig. 4, Table 1). (5) HT22 cells express the mRNA, protein, and physiological binding properties of D4 dopamine receptors (Fig. 7, Tables 2, 3).

It has been suggested that the neuroprotective effects of catecholamines, including dopamine, epinephrine, and norepinephrine are attributable to their antioxidant activities and are not receptor-mediated (Grünblatt et al., 1999; Noh et al., 1999). In the above experiments, it is indeed shown that many catecholamines have antioxidant activity, and that antioxidant activity may be part of the neuroprotective effect of apomorphine. However, D4 receptor mechanisms are more predominant than

antioxidant activities because the protective effect of apomorphine, which is a relatively good antioxidant, is the same as that of apocodeine, which is a relatively poor antioxidant, and stronger than catecholamines, which are also good antioxidants (Table 1). In addition, the D4 agonist PD168077 has no antioxidant activity but is very protective.

It is also known that exogenous dopamine can be neurotoxic. Dopamine is degraded to hydrogen peroxide and dihydroxyphenylacetaldehyde by monoamine oxidase or spontaneously oxidizes to form quinones, semiquinones, and again hydrogen peroxide. Several of these products can lead to the generation of reactive oxygen species such as hydroxyl radicals. Therefore, there has been considerable interest in the potential role of dopamine in CNS ischemia and trauma. Indeed it has been established in several *in vivo* models that dopamine is involved in the cell death pathway. For example, when brain lesions are caused by malonate or 3-nitropropionic acid, two reagents that inhibit energy metabolism, the experimental reduction of dopamine greatly diminishes the extent of damage (Reynolds et al., 1998; Ferger et al., 1999). These results are not, however, at odds with ours because the nature of the insults is quite different (energy deprivation versus oxidative stress) and the levels of multiple toxic agents released from both dying nerve and activated glia are likely to be much higher in severely traumatized CNS tissue than in oxidatively stressed nerve cell cultures. The significance of the dopamine D4 receptor activation may be that it protects cells from gradual changes in low level oxidative stress that occur in mild pathological insults and aging.

The mouse dopamine D4 receptor mRNA is found in the hippocampus and other brain regions, and also in some peripheral tissues such as the adrenal gland and the testes (Van Tol et al., 1991). It is not found in other peripheral tissues, including liver and kidney. Our RT-PCR data from mouse tissues (Fig. 7*A*) are consistent with the published data with respect to the regional specificity of this receptor and size of its mRNA. They also demonstrate that HT22 cells express dopamine D4 receptor mRNA. The mouse D4 receptor has 387 amino acids with a calculated molecular weight of 41,468 (Fishburn et al., 1995; Suzuki et al., 1995). This molecular weight is smaller than the estimated molecular weight from the western blots in this manuscript but is consistent with the apparent molecular weight of the dopamine D4 receptor published by others, probably because of glycosylation (Suzuki et al., 1995; Lanau et al., 1997).

Binding assays also show that dopamine D4 receptors are expressed in the HT22 cells and that apomorphine and apocodeine bind the receptors with higher affinities than dopamine. These data agree with those that show that although apomorphine is a nonselective dopamine receptor agonist, its affinity for D4 receptors is severalfold higher than that of dopamine (Seeman and Van Tol, 1994). In addition, the affinities of apomorphine and apocodeine for the D4 dopamine receptor in HT22 cells are the same as the dopamine D4 antagonist U101958 and higher than the D4 agonist PD168077. These data show that apomorphine, apocodeine, and dopamine can all act as dopamine D4 agonists.

A great deal is understood about the programmed cell death pathway initiated by glutamate in HT22 cells and primary cortical neurons (Maher and Davis, 1996; Murphy et al., 1989; Li et al., 1997a,b; Tan et al., 1998a,b). This knowledge makes it possible to determine at which point in the pathway the drug-induced inhibition of cell death occurs. There are two features of this pathway that are relevant to apomorphine protection. First, maximum ROS production requires the influx of extracellular Ca^{2+} (Li et al., 1997b; Tan et al., 1998a). Second, the influx of Ca^{2+} is initiated by an accumulation of intracellular cGMP via the activation of soluble guanylate cyclase (Li et al., 1997b). The data presented here show that apomorphine, apocodeine, and dopamine inhibit the cell death program at the level of cGMP-gated Ca^{2+} influx. In the presence of these compounds, ROS are elevated but do not reach their maximum levels (Fig. 5). This result is identical to that observed when the synthesis of cGMP is blocked, preventing Ca^{2+} influx, or when the Ca^{2+} channel is blocked by cobalt (Li et al., 1997b; Tan et al., 1998a). The data are therefore consistent with the block being associated with Ca^{2+} influx, a conclusion confirmed by Ca^{2+} imaging (Fig. 5). To more precisely localize the site of protection, it was asked whether apomorphine inhibits cell death that is caused by elevated intracellular cGMP. The elevation of cGMP and the resultant opening of the cGMP-gated Ca^{2+} channels are both necessary and sufficient to cause cell death in oxidative glutamate toxicity (Li et al., 1997b). Figure 6 shows that apomorphine, apocodeine, and dopamine all protect cells from elevated cGMP with a time of action indistinguishable from that of cobalt. Therefore, it is most likely that the activation of D4 receptors inhibits a step downstream of cGMP, probably at the Ca^{2+} channel. Indeed, it has been suggested that the activation of dopamine D4 receptors reduces Ca^{2+} currents (Sokoloff and Schwartz, 1995). The present study shows that apomorphine, apocodeine, and dopamine protect cells from oxidative stress-induced cell death by the inhibition of Ca^{2+} channels through the activation of dopamine D4 receptors.

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Original Contribution

FLAVONOIDS PROTECT NEURONAL CELLS FROM OXIDATIVE STRESS BY THREE DISTINCT MECHANISMS

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Abstract—Flavonoids are a family of antioxidants found in fruits and vegetables as well as in popular beverages such as red wine and tea. Although the physiological benefits of flavonoids have been largely attributed to their antioxidant properties in plasma, flavonoids may also protect cells from various insults. Nerve cell death from oxidative stress has been implicated in a variety of pathologies, including stroke, trauma, and diseases such as Alzheimer's and Parkinson's. To determine the potential protective mechanisms of flavonoids in cell death, the mouse hippocampal cell line HT-22, a model system for oxidative stress, was used. In this system, exogenous glutamate inhibits cystine uptake and depletes intracellular glutathione (GSH), leading to the accumulation of reactive oxygen species (ROS) and an increase in Ca^{2+} influx, which ultimately causes neuronal death. Many, but not all, flavonoids protect HT-22 cells and rat primary neurons from glutamate toxicity as well as from five other oxidative injuries. Three structural requirements of flavonoids for protection from glutamate are the hydroxylated C3, an unsaturated C ring, and hydrophobicity. We also found three distinct mechanisms of protection. These include increasing intracellular GSH, directly lowering levels of ROS, and preventing the influx of Ca^{2+} despite high levels of ROS. These data show that the mechanism of protection from oxidative insults by flavonoids is highly specific for each compound. © 2001 Elsevier Science Inc.

Keywords—Flavonoids, Oxidative stress, Glutamate toxicity, Vitamin E, Reactive oxygen species, Mitochondria, Glutathione, Neuronal cells, Quercetin, Buthionine sulfoximine, Apoptosis, Free radicals

INTRODUCTION

Flavonoids (Fig. 1) are a family of diphenylpropanes found ubiquitously in fruits and vegetables as well as in food products and beverages derived from plants such as olive oil, tea, and red wine. The flavonoid content in fruits and vegetables can be as high as 300 mg/kg fresh weight [1–3] and humans consume between 20 and 80 mg flavonoids per day, an intake higher than that for vitamin E [4]. Flavonoids are also considered to be the active ingredients in some medicinal plants [5,6]. Because ingested flavonoids enter the plasma [7–10] to elevate the redox and antioxidant levels [11,12], the effects of flavonoids may be physiologically significant. Indeed, flavonoids and other plant-derived polyphenolic compounds have recently captured public interest because flavonoids in tea, fruits, and vegetables reduce the risk of cardiovascular diseases [13,14]. Similarly, what is known as the "French Paradox" points to possible benefits of the Mediterranean diet, which includes high amounts of fresh fruits and vegetables as well as red wine [15].

These physiological benefits of flavonoids are generally thought to be due to their antioxidant and free radical scavenging properties, even though flavonoids display other biological activities [16–18]. The efficacy of flavonoids has been studied most extensively in cell-free systems in which reactive oxygen species (ROS) are produced either chemically [19,20] or by radiolysis [21], and the elimination of ROS by flavonoids is monitored directly or by measuring peroxidation levels of lipids [22–24] or LDL [7,25]. These experiments test the ability of flavonoids to act as antioxidants in an aqueous environment and have been very successful in elucidating relationships between activity and structure of flavonoids in solution [7,26]. It is difficult, however, to extend these results to cells that are under oxidative

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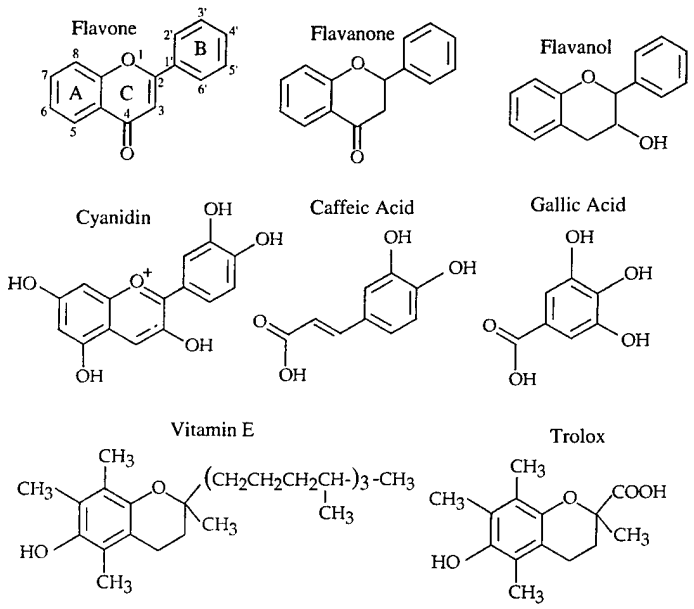

Fig. 1. Chemical structures of flavonoids and phenolic compounds used in this study. The position of hydroxyl groups and common names are listed in Table 1.

stress because the cellular structure encompasses both hydrophilic and hydrophobic compartments such as the cytoplasm and the various membrane systems, respectively.

While the sources of ROS vary in biological systems, significant levels of ROS are produced within cells [27]. For example, mitochondria produce ROS during normal respiration and mitochondrial derived ROS are required for many forms of cell death [28–31]. In neurons and other cell types, extracellular glutamate triggers oxidative stress and subsequent programmed cell death as the consequence of ROS generated from mitochondria [32–37]. Cellular markers and the precise time course of this cascade have been studied extensively in the mouse hippocampal cell line HT-22 [30,38–42]. These cells lack ionotropic glutamate receptors, and glutamate in this system inhibits cystine transport and leads to GSH depletion. Subsequently, ROS produced by mitochondria accumulate to levels 50- to 100-fold higher than the control, causing an increase in Ca^{2+} influx, and finally cell death [30,43]. The importance of oxidative stress in this cascade is supported by the observation that the exogenous addition of an antioxidant such as vitamin E protects cells from glutamate [30,33,38]. Therefore, this system was used to test the protective efficacy of plant-derived flavonoids against cellular oxidative stress.

We show that many flavonoids and related polyphenolic compounds protect HT-22 cells and rat primary neurons from oxidative stress caused by glutamate. The relationship between structure and protective efficacy of flavonoids was also determined. Additionally, flavonoids protect neuronal cells from oxidative injury caused by homocysteic acid (HCA), cystine deprivation, buthionine sulfoximine (BSO), hypoglycemia, and hydrogen peroxide (H_2O_2). Finally, three distinct mechanisms of protection by flavonoids can be identified: the alteration of GSH metabolism, quenching of ROS, and the inhibition of Ca^{2+} influx that signals the last step in the cell death cascade induced by glutamate.

MATERIALS AND METHODS

Flavonoids were purchased from Alexis (San Diego, CA, USA), Aldrich (Milwaukee, WI, USA), or CalBiochem (San Diego, CA, USA). 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-dA), dihydrorhodamine, Fluo-3, Pluronic 127, indo-1, and Fura Red were purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

Cell culture

Fetal bovine serum (FBS) and dialyzed FBS were from Irvine Scientific (Irvine, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was made according to the original procedure [44]. HT-22 cells [38,39] were derived from the mouse hippocampus [45], and were grown on tissue culture dishes (Falcon, Indianapolis, IN, USA) in DMEM supplemented with 10% FBS. Pancreatin (Life Technologies, Rockville, MD, USA)

was used to dissociate cells from culture dishes. Short-term primary cortical neurons from 17 d old rat embryos were prepared according to Abe and Kimura [46]. The primary cells were used for experiments within 3 d after plating. Except where noted, all experiments were done with HT-22 cells.

Cytotoxicity assay

Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to a standard procedure [47–49]. Results obtained from the MTT assay directly correlated with the extent of cell death as confirmed visually by trypan blue staining. The culture medium for both primary neurons and HT-22 both contained 260 μM cystine and 1 mM Mg^{+2}. H_2O_2 toxicity was performed according to conditions established previously [30,42].

Total intracellular GSH/GSSG

Cells were washed twice with ice-cold PBS, collected by scraping, and lysed with 3% sulfosalicylic acid. Lysates were incubated on ice for 10 min and supernatants were collected after centrifugation in an Eppendorf microfuge. Upon neutralization of the supernatants with triethanolamine, the concentration of total glutathione (reduced and oxidized) was determined by the method described originally by Tietze [50] and modified by Griffith [51]. Pure GSH was used to obtain the standard curve. The protein content of each sample was determined using a kit from Pierce (Rockford, IL, USA) with BSA as a standard.

Reactive oxygen species (ROS) levels

Intracellular accumulation of ROS was determined with H_2DCF-dA [52]. This nonfluorescent compound accumulates within cells upon de-acetylation. H_2DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF) [53]. HT-22 cells were dissociated from tissue culture dishes with pancreatin in DMEM in the presence of 10 μM H_2DCF-dA for 10 min at 37°C, washed once with room temperature DMEM (without phenol red) supplemented with 2% dialyzed FBS, and resuspended in 750 μl of the same solution containing 2 μg/ml propidium idodide (PI). The use of pancreatin did not affect the outcome of flow cytometric experiments as confirmed by fluorescence microscopy. Flow cytometric analysis was performed using a FACScan instrument (Becton-Dickinson, San Jose, CA, USA) with the excitation wavelength (λ_{ex}) of 475 nm and the emission wavelength (λ_{em}) of 525 nm. Data were collected in list mode on 10,000 cells after gating only for characteristic forward versus orthogonal light scatter and low PI fluorescence to exclude dead cells. Median fluorescence intensities of control and test samples were determined with CellQuest software (Becton-Dickinson).

Intracellular calcium

The intracellular level of Ca^{2+} was determined using Fluo-3 acetoxymethylester (AM) or Fura Red as described elsewhere [30,42,54]. The membrane permeable Fluo-3 AM is converted to Fluo-3 upon de-acetylation within the cell and Fluo-3 increases its green fluorescence upon Ca^{2+} binding. Cells were loaded with 0.5 μM Fluo-3 AM in the presence of 0.005% Pluronic 127 for 20 min at 37°C, washed once with room temperature DMEM (without phenol red) supplemented with 2% dialyzed FBS, and resuspended in 4 ml of the same solution containing 2 μg/ml PI. A Nikon light/fluorescence microscope equipped with 32 mm water-immersible objective lens was used to determine Fluo-3 fluorescence and photographed with Kodak Ektachrome 400HC.

Determination of the trolox equivalent activity concentration (TEAC)

Values of TEAC for flavonoids and other phenolic compounds were determined according to Rice-Evans and Miller [55]. Briefly, the inhibition of the absorbance of the radical cation formation of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) was monitored at 7.5 min in a Dulbecco's phosphate buffered saline (PBS) containing 150 μM ABTS, 2.5 μM metmyoglobin, and 75 μM H_2O_2 at 734 nm. The TEAC value of an antioxidant was calculated experimentally using a 0.3 mM Trolox solution and normalizing the data to 1.0 mM Trolox (i.e., 0.3 mM Trolox was used in the experiments and the data multiplied by 3.3 to convert to 1 mM equivalents).

Statistical analysis

Experiments presented were repeated at least twice with triplicate samples. The data are presented as means ± SEM.

RESULTS

Protection from glutamate toxicity by flavonoids

The mouse hippocampal cell line HT-22 has been used to elucidate sequential cellular events during the programmed cell death cascade triggered by glutamate toxicity [30,38,42]. HT-22 cells lack ionotropic glutamate receptors that could mediate excitotoxicity, and are

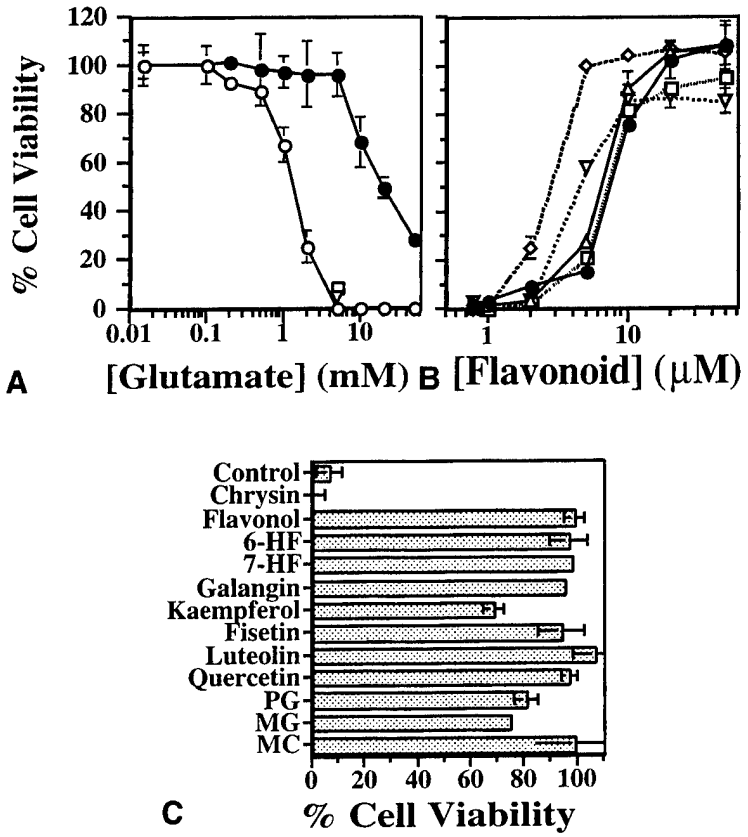

Fig. 2. Cytotoxic response of HT-22 cells to glutamate and protection by flavonoids. Exponentially dividing HT-22 cells were dissociated with pancreatin and plated into a 96 well microtiter plate in 100 μl DMEM with 10% dialyzed FBS. (A) 12 h later cells were exposed to the indicated concentrations of glutamate in the presence of no flavonoids (○), 10 μM galangin (●), 10 μM chrysin (1 point, 5 mM) (▽) or 10 μM catechin (1 point, 5 mM) (□) for 24 h and cell viability was assessed with the MTT assay as described in Materials and Methods. (B) Cell viability was assessed as above in the presence of 5 mM glutamate and the indicated concentrations of galangin (●), flavonol (Δ), 6-HF (□), luteolin (△), or quercetin (◇). (C) Rat primary cortical neurons were exposed to 5 mM glutamate for 24 h in the presence of 10 μM flavonoids as indicated. Cell viability was determined with the MTT assay.

completely killed by 5 mM glutamate in 24 h with a half maximal concentration 1.5 mM (Fig. 2A). This toxicity is via the inhibition of cystine uptake by glutamate, resulting in the depletion of glutathione (GSH), and a form of programmed cell death called oxidative glutamate toxicity [33]. To determine if flavonoids are effective protectants against this form of oxidative injury, HT-22 cells were exposed to various concentrations of glutamate for 24 h in the presence of 10 μM of the flavonoids galangin, chrysin, or catechin, and cell viability was determined by the MTT assay. Galangin protects HT-22 cells from glutamate, but chrysin and catechin are ineffective (Fig. 2A). To determine the effective concentrations of flavonoids, HT-22 cells were exposed to 5 mM glutamate in the presence of various concentrations of a flavonoid and cell viability was determined as above. Galangin, flavonol (3-hydroxyflavone), and 6-hydroxyflavonol (6-HF) all have the effective half maximal concentrations (EC_{50}) of about 7 μM, and the maximum protection is observed with concentrations higher than 10 μM (Fig. 2B). Quercetin and luteolin have the EC_{50} of 2.2 μM and 4 μM, respectively (Fig. 2B). The protection by these flavonoids is not transient: the neuronal cells are viable in toxic doses of glutamate for at least 4 d if protective flavonoids are continuously present (data not shown).

Unexpectedly, the number of hydroxyl groups in a flavonoid did not correlate with the protective efficacy [26]. For example, flavonol with one hydroxyl group is as effective as galangin (three hydroxyl groups) while catechin (five hydroxyl groups) is totally ineffective (Fig. 2A and B). Therefore, the efficacy of several commercially available flavonoids and phenolics was determined to elucidate structural requirements for the protection of HT-22 cells from glutamate toxicity (Table 1). Some flavonoids (α- and β-naphthoflavone) and flavonoid analogs (chalcones and coumarins) are not protective (data not shown), while other phenolic compounds are quite active (Fig. 1; Table 1). The results and the structural requirements for protection will be summarized in the Discussion. The flavonoids that protect HT-22 cells from

Table 1. The Protective Efficacy of Various Flavonoids

Flavonoid[a]	Free hydroxyl positions	Common name	EC_{50} (μM)	TEAC[b] (mM)
Flavone and flavonol	—		No[c]	0.30 ± 0.10
	3	flavonol	6	1.06 ± 0.18
	3-methoxy		No	−0.01 ± 0.07
	6		No	0.95 ± 0.18
	6-methoxy		No	−0.04 ± 0.05
	7		No	−0.04 ± 0.02
	3,6		6	2.06 ± 0.20
	3,7		6	1.65 ± 0.12
	5,7	chrysin	No	2.52 ± 0.12
	3,5,7	galangin	6	2.08 ± 0.11
	5,6,7	baicalein	1	1.22 ± 0.17
	4′,5,7	apigenin	No	2.80 ± 0.50
	3,4′,5,7	kaempferol	10	1.45 ± 0.08
	3′,4′,5,7	luteolin	5	2.48 ± 0.23
	3,3′,4′,7	fisetin	3	2.80 ± 0.06
	3,3′,4′,5,7	quercetin	3	4.84 ± 0.45
	3,3′,4′,5,7[d]	rutin	No	2.67 ± 0.24
	2′,3,4′,5,7	morin	70	2.60 ± 0.24
	3,3′,4′,5,5′7	myricetin	No	3.08 ± 0.46
Isoflavone	4′,5,7	genistein	No	2.96 ± 0.49
Flavonone	—		No	0.01 ± 0.12
	6		No	1.47 ± 0.14
	4′,5,7	naringenin	No	2.48 ± 0.24
	3,3′,4′,5,7	taxifolin	No	3.09 ± 0.58
Flavanol	3,3′,4′,5,7	catechin	No	3.42 ± 0.43
	3,3′,4′,5,7	epicatechin	No	3.16 ± 0.58
Anthocyanidin	3,3′,4,29,5,7	cyanidin	No	3.63 ± 0.65
Vitamin E			0.2	1.1 ± 0.14
Vitamin E acetate			1.5	ND
Vitamin E succinate			12	ND
Trolox			50	1
Probucol			10	0.01 ± 0.15
Caffeic acid	3,4		No	1.20 ± 0.05
Methyl caffeate (MC)	3,4		0.5	1.21 ± 0.15
Gallic acid	3,4,5		No	2.69 ± 0.41
Methyl gallate (MG)	3,4,5		8	2.14 ± 0.30
Propyl gallate (PG)	3,4,5		0.2	2.42 ± 0.08
Catechin gallate (CG)			No	5.12 ± 0.55
Resveratrol	3,4′,5		8	2.88 ± 0.15
PBN[e]			No	ND

Half maximal effective concentrations (EC_{50}) were determined by exposing HT-22 cells to 5 mM glutamate in the presence of a flavonoid and cell viability was assessed as in Fig. 2. Additionally, TEAC values were determined as in Materials and Methods in two triplicate experiments and means ± SEM were reported.

[a] See Fig. 1 for chemical structures.

[b] TEAC is defined as the concentration of Trolox solution equivalent to a standard concentration of the compound in question and the mean ± SEM was presented.

[c] No—These compounds did not protect cells significantly from 5 mM glutamate at 50 μM or lower concentrations.

[d] Rutin is quercetin with the substitution on the C3 hydroxyl group. ND = not determined.

[e] N-*tert*-butyl-α-phenylnitrone.

glutamate toxicity are also effective protectants of primary rat cortical neurons from glutamate toxicity (Fig. 2C), showing that the response of HT-22 neurons to oxidative stress is typical of cortical neurons.

Protection from other forms of oxidative stress by flavonoids

It was asked next if flavonoids protect HT-22 cells from other forms of oxidative injury. Cystine deprivation caused by excess glutamate is also induced by homocysteic acid (HCA) or by excluding cystine from the culture medium [33,39,42]. In contrast, BSO lowers levels of intracellular GSH by inhibiting the GSH synthetic enzyme γ-glutamylcysteine synthetase (γ-GCS) [56]. Fifty μM BSO lowers GSH levels to near zero in HT-22 cells [30,40,41], as does cystine deprivation and 2 mM HCA, resulting in neuronal death [42,43]. In all of these cases galangin at 10 μM protects HT-22 cells (Fig. 3A). All of the other flavonoids that protect HT-22 cells from glutamate are also effective against HCA, cystine deprivation, and BSO (data not shown).

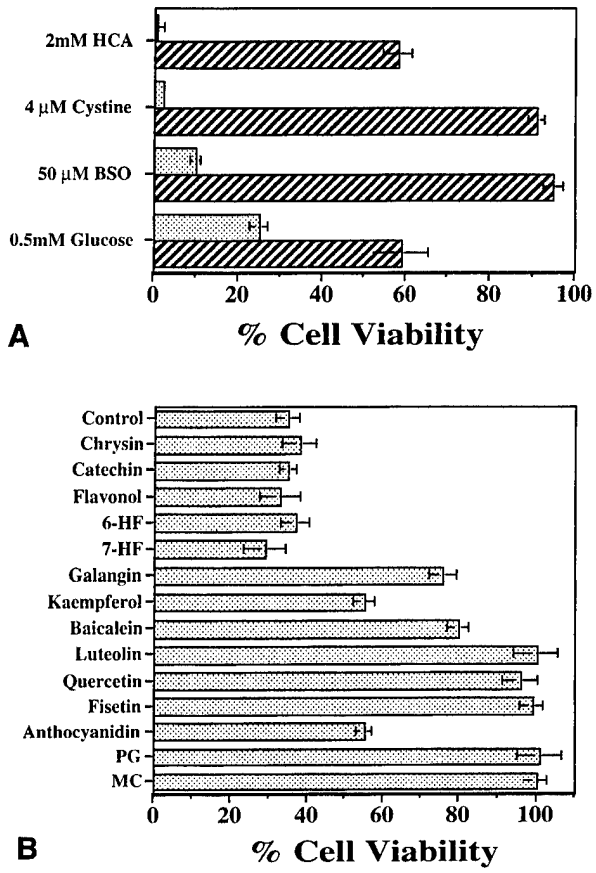

Fig. 3. Protective effects of flavonoids to oxidative stress. HT-22 cells were plated into a 96 well microtiter plate as in Fig. 2. 12 h later the cells were exposed to the indicated conditions for 24 h in the presence (hatched bars) or absence (dotted bars) of 10 μM galangin (A). Cells were exposed to 1 mM H_2O_2 for 8 h in the presence of a flavonoid (10 μM) as indicated (B). The cells were then cultured for an additional 16 h in fresh media and cell viability was determined by the MTT assay.

Oxidative stress is also induced during glucose starvation, or hypoglycemia [57,58]. HT-22 cells die when the glucose concentration of the culture media is decreased from the normal 50 mM to 0.5 mM [49]. Galangin increases cell viability from 20 to 60% in hypoglycemic injury (Fig. 3A). Other flavonoids found to be protective in glutamate toxicity (flavonol, 6-HF, 7-HF, galangin, baicalein, kaempferol, luteolin, fisetin, and quercetin) also effectively protect HT-22 cells from hypoglycemia and the oxidative injuries listed above (data not shown).

The direct addition of the peroxidizing agent H_2O_2 also induces neuronal death. Therefore, the protective efficacy of flavonoids was assessed by exposing HT-22 cells to H_2O_2 for 24 h. Luteolin, quercetin, fisetin, propyl gallate (PG), and methyl caffeate (MC) almost completely block H_2O_2 toxicity (Fig. 3B). Galangin and baicalein protect HT-22 cells to 70 to 80% viability,

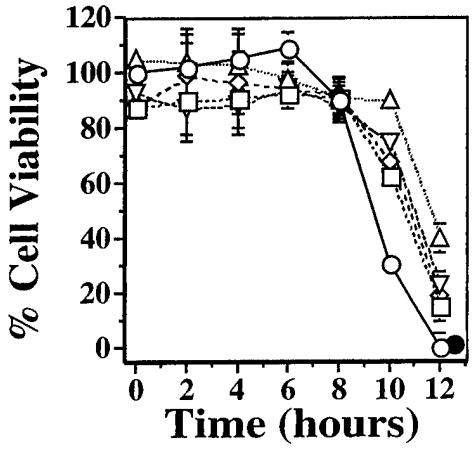

Fig. 4. Time course of glutamate toxicity. HT-22 cells were plated into 96 well microtiter plates as in Fig. 2 and 12 h later exposed to 5 mM glutamate. 24 h glutamate exposure resulted in less than 3% survival (●). For one sample (○), glutamate was removed at the indicated time after the glutamate addition and cell viability was determined 24 h later. For other samples, 10 μM flavonoids were added at the indicated time after the glutamate addition. In all cases cell viability was assessed by the MTT assay 24 h after the glutamate addition. The following flavonoids were used: flavonol (△), 6-HF (□), baicalein (▽), and quercetin (◇).

while kaempferol and anthocyanidin have marginal effects. Other flavonoids such as flavonol, catechin, and chrysin are ineffective against the H_2O_2 toxicity. From the above results, it can be concluded that many flavonoids protect HT-22 cells from several different forms of oxidative stress.

Time course of protection

Because oxidative glutamate toxicity initiates a well-defined temporal cascade of obligatory and sequential events, the mechanism of protection by a compound may be deciphered by the latest time when the compound is still protective after the addition of glutamate [30,38]. Therefore, flavonoids were added to HT-22 cells at 2 h intervals following glutamate, and the cells were then incubated for a total of 24 h. The MTT assay in Fig. 4 shows that the withdrawal of glutamate before 8 h into the cascade prevents subsequent cell death, but the cells cannot reverse the death program after 8 h ("the point of no return"). Effective flavonoids protect cells as late as 10 h after the glutamate addition. These results suggest a latent action by flavonoids. To further determine the protective mechanism by flavonoids, cellular metabolic markers—GSH levels, ROS levels, and Ca^{2+} influx—were examined.

Glutathione levels

Glutamate inhibits cystine uptake, causing the total loss of the cellular GSH within 8 h [42,43]. Cells can be

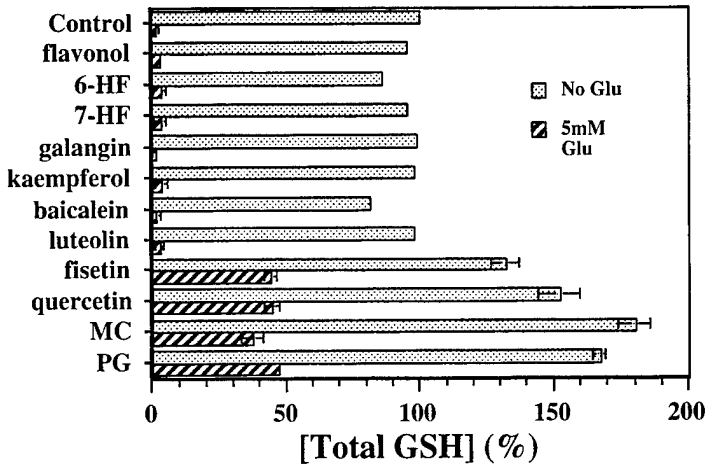

Fig. 5. Effects of flavonoids on cellular levels of glutathione. HT-22 cells were treated with the indicated flavonoids (10 μM) in the absence (dotted bars) or presence (hatched bars) of 5 mM glutamate for 10 h and cellular levels of total glutathione (GSH) were measured as described in Materials and Methods. The GSH level of the control sample (56 ± 1.7 nmol/mg protein) was taken as 100%. The results are the means of triplicate determinations ± SEM from two to three independent experiments.

rescued from glutamate toxicity by mechanisms either dependent upon or independent of GSH metabolism. For example, antioxidants such as vitamin E protect neuronal cells from oxidative glutamate toxicity without affecting the intracellular GSH levels [33,38]. In contrast, dihydroxyphenylglycine, an agonist of Group I metabotropic glutamate receptors, protects neurons by upregulating GSH [49]. To determine if the protection by flavonoids is via GSH metabolism, HT-22 cells were treated with 5 mM glutamate in the presence or absence of a flavonoid for 10 h, harvested, and total GSH determined. As shown in Fig. 5A, some protective flavonoids such as flavonol, galangin, and baicalein do not increase the basal levels of GSH, nor do they prevent GSH loss caused by glutamate. In contrast, quercein and fisetin, as well as PG and MC, increase basal levels of GSH by 30 to 80% relative to untreated cells. Even in the presence of 5 mM glutamate HT-22 cells treated with these compounds maintain 40% to 50% of the GSH level in the untreated cells. It has been shown previously that cells survive glutamate toxicity if the cellular GSH level is more than 15 to 20% of the control level [42,49]. Therefore, quercetin, fisetin, PG, and MC protect HT-22 cells from glutamate toxicity by altering GSH metabolism.

Reactive oxygen species

The loss in cellular GSH up to 85% of the control level causes only a 5- to 10-fold increase in levels of ROS [30]. A greater GSH loss, however, stimulates mitochondria to produce a 100-fold increase in ROS, resulting in cell death [30]. This is clearly shown in the sample treated with 5 mM glutamate and the nonprotective flavonoid chrysin for 11 h (Fig. 6A). A similar analysis was performed for the group of protective flavonoids and the fold increase in the median fluorescence intensity in the presence of 5 mM glutamate with respect to that of the basal level was determined (Fig. 6B). Therefore on the basis of their effect on cellular ROS and GSH, protective flavonoids can be roughly classified into three groups: the quercetin type, the flavonol type, and the galangin type. HT-22 cells treated with glutamate in the presence of the quercetin type (quercetin, fisetin, PG, and MC) accumulate a relatively low level of ROS (Fig. 6B), possibly because these compounds maintain the cellular GSH above the critical level for the explosive generation of ROS (Fig. 5). In contrast, the treatment with the flavonol type (flavonol, 6-HF, and 7-HF) does not prevent the accumulation of ROS in glutamate toxicity. The high level of fluorescence was not due to the autofluorescence of the flavonoids because cells treated only with flavonol did not have a high background (data not shown). Cells treated with the flavonol type also show consistently elevated levels of ROS, even though the cells were at least 90% viable throughout the experiment and for at least 30 h thereafter (Fig. 2A). These results were confirmed by using fluorescent probes dihydrorhodamine 123 or dihydroethidium for ROS detection (data not presented). Finally, the galangin type (galangin, baicalein, kaempferol, and luteolin) show an increase in ROS of less than 10-fold, which is similar to vitamin E, but also have low levels of GSH (Fig. 5) [59].

Antioxidative efficacy

The activity of flavonoids is generally explained by their antioxidative efficacy. But because the flavonol

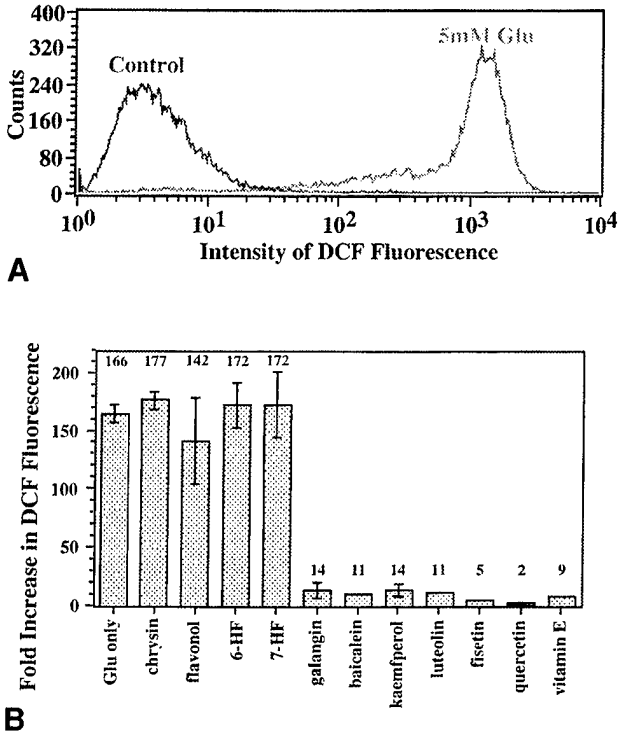

Fig. 6. Effects of flavonoids on ROS levels. HT-22 cells were treated with 5 mM glutamate and chrysin (A) for 11 h and the sample was loaded with H_2DCF-dA and processed as described in Materials and Methods. Levels of ROS were measured with DCF fluorescence and channels of fluorescence intensity were plotted against the counts in each channel. (B) Levels of ROS were measured in the presence of 10 μM flavonoids as above and the fold increase in the median DCF fluorescence intensity with respect to that of the control was plotted. The results are the means of duplicate determinations ± SEM from three independent experiments. Numbers listed above the bar graphs are the levels of the fold increase in DCF fluorescence compared to the control level.

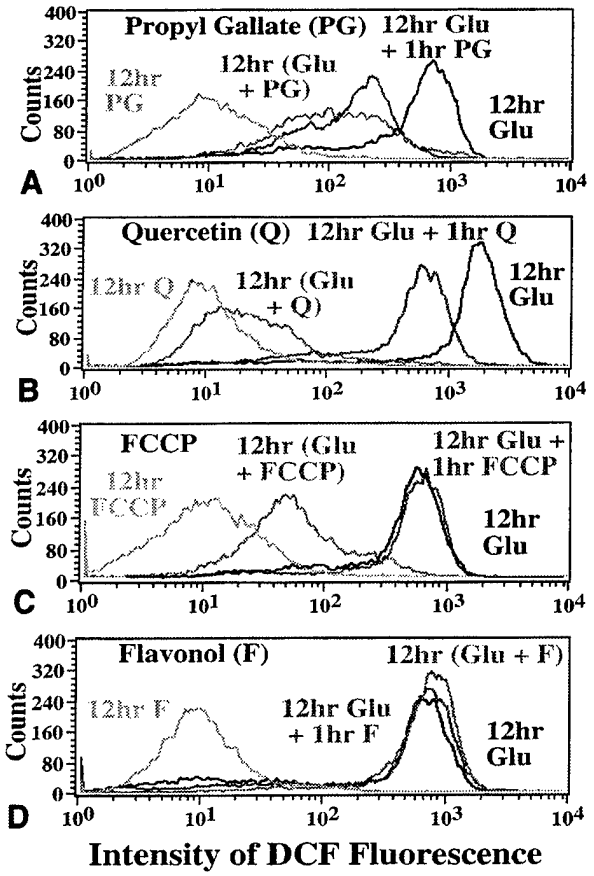

Fig. 7. Antioxidative effects of flavonoids. (A) HT-22 cells were treated either with 10 μM propyl gallate (PG) only (12 h PG − green) or with 5 mM glutamate for 12 h in the absence (12 h Glu − black) or presence of 10 μM PG (12 h (PG + Glu) − blue). In another set of samples, HT-22 cells were treated with 5 mM glutamate for 11 h and 10 μM PG was added for 1 h (12 h Glu + 1 h PG − red). Then, levels of ROS were measured using DCF fluorescence as in Fig. 6 and channels of fluorescence intensity were plotted against the counts in each channel. (B) Same as above except with 10 μM quercetin (Q). (C) Same as above except with 10 μM FCCP. (E) Same as above except with 10 μM flavonol (F).

type (flavonol, 6-HF, and 7-HF) did not prevent the increased accumulation of ROS in HT-22 cells after glutamate treatment, their antioxidative properties were studied using a well-established procedure to determine Trolox Equivalent Activity Concentration (TEAC) [55]. In this procedure, a substance is compared to 1 mM Trolox, a water-soluble vitamin E analog, in its ability to suppress the radical cation of ABTS in an aqueous solution [20]. In addition to TEAC values for some flavonoids reported previously [26], we determined values for several others (Table 1). The TEAC values of many flavonoids do not correlate with the protective efficacy against cellular oxidative stress (Table 1).

The discrepancy between TEAC values and the protective efficacy of flavonoids observed in Table 1 may be attributable to different accessibility of flavonoids to ROS in the two systems. While TEAC values reflect the ability of flavonoids to quench free radicals in aqueous solution, in glutamate toxicity the generation and accumulation of ROS take place in mitochondria and in the cytoplasm. Therefore, to determine the ability of exogenous flavonoids to quench ROS in cells, HT-22 cells were treated with glutamate for 11 h, the time point at which the cells are alive but accumulate high levels of ROS (Fig. 6A; [30]). The flavonoid was then added to the cells in the presence of glutamate for an additional 1 h and processed for the determination of DCF fluorescence as in Fig. 6. HT-22 cells accumulate a high level of ROS after 12 h incubation with 5 mM glutamate (Fig. 7A, "12hr Glu") compared to the control sample treated only with 10 μM PG (Fig. 7A, "12hr PG"). HT-22 cells treated with PG and 5 mM glutamate accumulate a lower level of ROS after 12 h (Fig. 7A, "12hr (Glu + PG)"; see, also Fig. 6B). That PG can also act as an antioxidant is shown by its ability to decrease accumulated levels of ROS within 1 h (Fig. 7A, "12hr Glu + 1hr PG"). Quer-

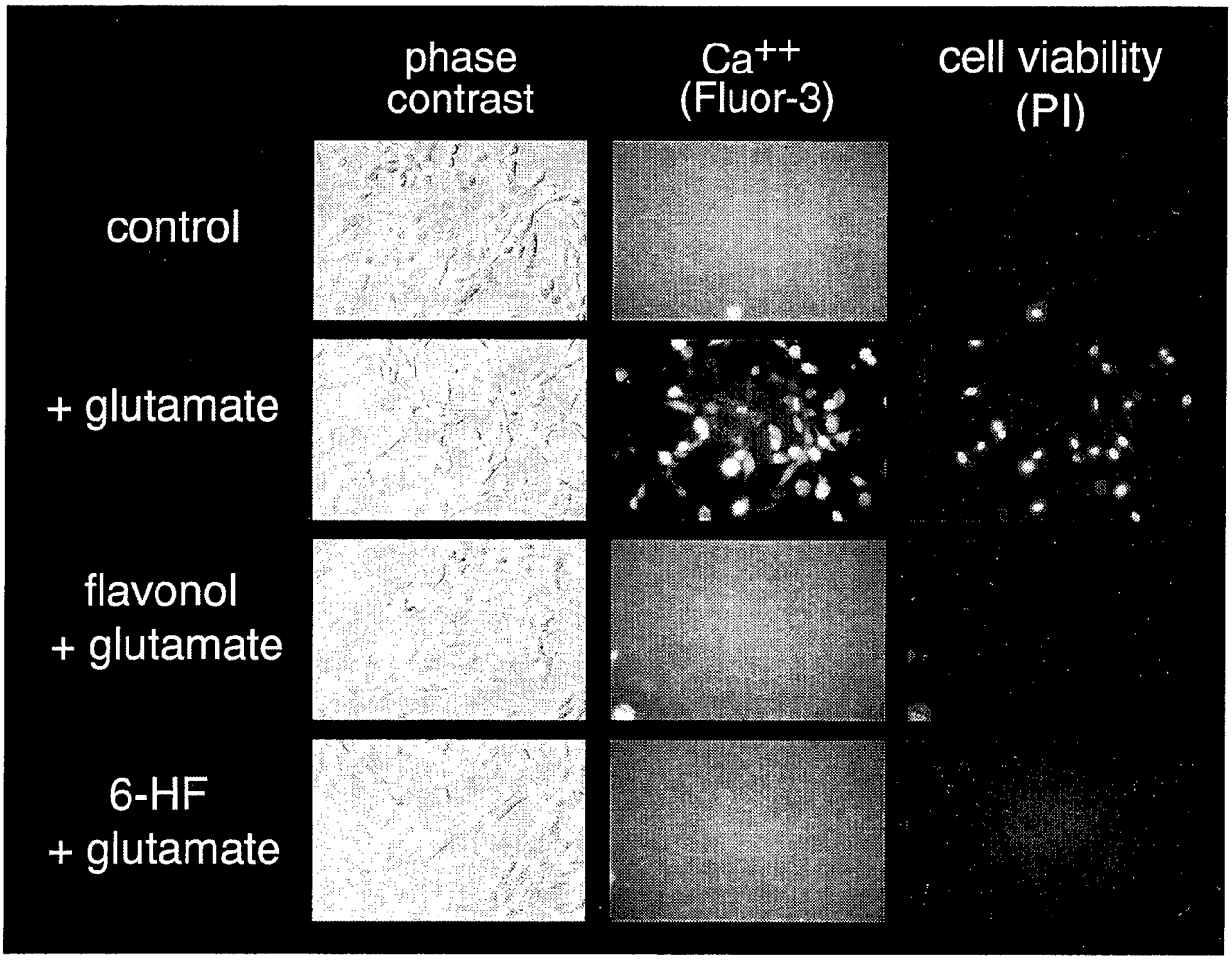

Fig. 8. Effects of flavonoids on Ca^{2+} influx caused by glutamate. HT-22 cells (4×10^5 cells) were seeded in 60 mm tissue culture dishes. 18 h later 5 mM glutamate and indicated flavonoids (10 μM) were added to the cells and incubated for 11 h. Cells were then loaded with 0.5 μM Fluo-3 AM in the presence of 0.005% Pluronic 127 as indicated in Materials and Methods. First column: phase contrast view. Second column: Ca^{2+} levels (Fluo-3 fluorescence). Third column: cell viability (PI fluorescence).

cetin (Fig. 7B) can also quench ROS, indicating the antioxidative property. Other protective flavonoids, baicalein, luteolin, galangin, kaempferol, and fisetin all acted as antioxidants in this system (data not shown).

In contrast, a mitochondrial uncoupler cyanide p-trifluoromethoxyphenylhydrazone (FCCP) decreases levels of ROS and protects HT-22 cells from glutamate toxicity only if added prior to "the point of no return" because mitochondria are the source of ROS in this system (Fig. 7C, "12hr (Glu + FCCP)") [30]. FCCP, however, cannot scavenge ROS if added after the generation of ROS (Fig. 7C, "12hr Glu + 1hr FCCP"). Finally, flavonol does not decrease the accumulated ROS under this condition (Fig. 7D, "12hr Glu + 1hr F"); the ROS level was similar to that in the cells treated with glutamate alone (Fig. 7D, "12hr Glu"). Similar results were obtained with 6-HF or 7-HF (data not shown). These results support the hypothesis that the flavonol type may protect HT-22 cells from oxidative stress not by quenching ROS but by affecting a further downstream step in the cellular metabolism in the glutamate toxicity cascade.

Ca^{2+} influx

In oxidative glutamate toxicity, a 100-fold increase in the intracellular ROS results in the elevation of cytosolic Ca^{2+}, which precedes cell death [30]. Because the flavonol type protects HT-22 cells from glutamate despite high ROS levels (Fig. 6B), it was asked if the surviving cells have elevated levels of cytosolic Ca^{2+}. The ratiometric calcium indicator Indo-1 could not be used due to the autofluorescence of some flavonoids [60]. Therefore, the intracellular Ca^{2+} was monitored with the membrane permeable Ca^{2+} specific fluorescence indicator Fluo-3. Control cells show negligible fluorescence. (Fig. 8; the first row). Cells treated with the flavonol type alone all showed a similar pattern of low intracellular Ca^{2+} (data

not shown). Five mM glutamate treatment for 9 h increases the intracellular Ca^{2+} in HT-22 cells ([30]; Fig. 8, second row), and causes cell death as indicated by PI staining (last column). In the presence of flavonol and 6-HF, the intracellular Ca^{2+} remains low and the cells did not die. Therefore, the flavonoids flavonol and 6-HF protect cells by preventing Ca^{2+} influx despite high intracellular levels of ROS. Similar results were obtained with 7-HF.

DISCUSSION

The above results show that some flavonoids protect neuronal cells from oxidative glutamate toxicity and other forms of oxidative injuries caused by cystine deprivation, BSO, hypoglycemia, and H_2O_2. Using glutamate toxicity as a well-characterized model of oxidative injury, we determined the structural requirements of flavonoids for efficacy of protection. Finally, flavonoids were shown to protect from oxidative stress by three distinct mechanisms: directly affecting GSH metabolism, acting as antioxidants, and maintaining low Ca^{2+} levels despite high levels of ROS.

Structural requirements for protection

Because flavonoids protect neuronal cells by three distinct mechanisms, it is difficult to derive at a single structure-activity relationship for different flavonoids. Nevertheless, three structural determinants required for protection can be deduced from Table 1: the presence of the hydroxyl group on the C3 position, an unsaturated C ring, and hydrophobicity. The requirement of the identical determinants has been described previously in aqueous and lipophilic cell-free systems [7,21,26,61] (see below).

Hydroxyl groups

On the A and C rings, the efficacy of protection is altered most dramatically by the hydroxyl group on the C3 position (see Fig. 1). Thus, flavonol is the only effective monohydroxyflavone. Furthermore, the C3 hydroxyl group converts the ineffective 6- or 7-hydroxyflavone to the protective dihydroxyflavones (EC_{50} = 6 μM). The conversion is attributable not to the number of the hydroxyl groups but to the hydroxyl placement on the C3, because chrysin with hydroxyl groups on C5 and C7 is totally ineffective. The same requirement may also affect the isoflavone genistein, which lacks the C3 hydroxyl group and is thus ineffective in protection. The C3 hydroxyl group cannot be methoxylated as evidenced by the ineffectiveness of 3-methoxyflavone. The importance of the C3 hydroxyl group is also observed in the antioxidant and free radical scavenging activities of flavonoids in the cell-free systems [21,26]. One subtle but significant difference between the two systems is that in the cell-free systems the C3 and C5 positions are considered to be identical in terms of charge dispersion, but their protective efficacy against glutamate is distinct [21]. Thus, chrysin (5,7-dihydroxyflavone) is an effective antioxidant (the TEAC value of 2.52), but an ineffective protectant against glutamate toxicity (Table 1). In contrast, 7-HF (3,7-dihydroxyflavone) is a poorer antioxidant (the TEAC value of 1.65) but a better protectant than chrysin. The difference may be attributable, among other factors, to the involvement of several mechanisms of protection, some of which do not depend on the antioxidant activity (see below).

Two exceptions to the requirement of the hydroxylated C3 are baicalein (5,6,7-trihydroxyflavone) and luteolin (3′,4′,5,7-tetrahydroxyflavone), both of which are protective. These exceptions may arise from the fact that they protect HT-22 cells from glutamate by inhibiting lipoxygenases, an enzyme involved in glutamate toxicity [41], although both of these flavonoids can act as antioxidants (Table 1; Figs. 6 and 7; see below).

On the B ring, the efficacy of protection is influenced only by the hydroxyl groups on both C3′ and C4′ positions (together they form the catechol o-dihydroxy structure). For example, flavonoids with the catechol structure are more protective than the C4′ position alone as seen in luteolin (compared to apigenin) and quercetin (compared to kaempferol) or the dihydroxyl groups on the C2′ and C4′ positions as in morin. The catechol structure is known to give excellent radical scavenging properties [21,26]. Hydroxyl groups on other positions on the B ring have no effects. For example, apigenin (4′,5,7-trihydroxyflavone) is as ineffective in protection, and chrysin (5,7-dihydroxyflavone) and both galangin (3,5,7-trihydroxyflavone) and kaempferol (3,4′,5,7-tetrahydroxyflavone) have a similar EC_{50}. Also, the requirement of these hydroxyl groups may be attributable to the inhibition of lipoxygenases as seen in analogs of flavonoids (chalcones) which inhibit 5-lipoxygenase when the hydroxyl groups are present in comparable positions [62].

Unsaturation

The unsaturation of the C ring in a flavonoid is essential for the protection from glutamate toxicity. This conclusion is based on the comparison of quercetin, taxifolin, and catechin, all of which contain five hydroxyl groups on the identical positions (3,3′,4′,5,7) (see Table 1 and Fig. 1). Quercetin with the unsaturated C ring is the only protective flavonoid of the three, and flavanones

(taxifolin and naringenin) and flavanols (catechin and epicatechin) are totally ineffective (Table 1). The exception to this rule is anthocyanidin, but this can be explained by the decreased hydrophobicity of this compound compared to other flavonoids as discussed below. The unsaturation of the C ring, which allows the electron delocalization across the molecule for the stabilization of the free radical, is also an important factor for antioxidants in the cell-free systems [21,26].

Hydrophobicity

In general, the more hydrophobic a flavonoid is, the more protective it is against glutamate toxicity. Thus, the decrease in hydrophobicity by glycosylation (for example, rutin), polyhydroxylation (myricetin), or ionization (cyanidin) inactivates corresponding protective flavonoids. The placement of the hydroxyl group on the C2′ also decreases hydrophobicity because it is ionized readily [63], resulting in decreased efficacy as seen in morin. The same requirement for high hydrophobicity can be further extended to other antioxidants and free radical scavengers. For example, the derivatization of vitamin E, MC, and PG to decrease hydrophobicity results in the decreased efficacy of protection against glutamate (Table 1). The fact that the spin trap N-tert-butyl-α-phenylnitrone (PBN) is not protective against glutamate may be also attributable to its charge. A hydrophobic antioxidant may easily enter the cytoplasm where ROS are generated and accumulate in glutamate toxicity. Hydrophobicity is also an important determinant of protective compounds in other forms of oxidative stress [64].

Because of the importance of hydrophobicity, TEAC values as defined by Rice-Evans [26] do not predict the efficacy of protection in glutamate toxicity. For example, cyanidin has the TEAC value higher than all other flavonoids except quercetin, but it is totally ineffective in protection against glutamate (Table 1). Also, vitamin E is the most effective of the vitamin E analogs in this system, while vitamin E and Trolox have similar TEAC values (Table 1). Structure-activity relationships of flavonoids from other studies in lipid peroxidation and lipophilicity [65], LDL oxidation [25], lipid peroxidation in membranes [24], inhibition of mitochondrial function [61], and metal chelation [66] also fail to predict their efficacy of protection in the cell-based glutamate toxicity assay. This failure may be attributable to the presence of both hydrophilic and hydrophobic compartments in cells such as the cytoplasm, the plasma membrane, and mitochondria. Therefore, oxidative glutamate toxicity may be a useful screening system to assess biological efficacy of compounds that may protect neurons and other cell types from oxidative stress.

Table 2. The Classification of Flavonoids by Protective Mechanisms

Protection type	Flavonoids and phenolic compounds	GSH metabolism	ROS Scavenger	Anti-Ca^{2+} influx
Flavonol	flavonol	−	−	+
	6-HF	−	−	+
	7-HF	−	−	+
Galangin	galangin	−	+	
	baicelein	−	+	
	kaempferol	−	+	
	luteolin	−	+	
Quercetin	quercetin	+	+	
	fisetin	+	+	
	vitamin E	−	+	
	methyl caffeate	+	+	
	propyl gallate	+	+	

The classification of flavonoids according to protective mechanisms is presented. Protective flavonoids were categorized into three types (flavonol, galangin, and quercetin) depending on the earliest cellular markers affected by the flavonoids in the glutamate toxicity cascade. Three other phenolic compounds are also presented for comparative purposes.

Mechanisms of protection

When HT-22 cells and primary cortical neurons lacking ionotropic glutamate receptors are exposed to glutamate the cells die via a programmed cell death pathway which involves, sequentially, a decrease in intracellular GSH, new protein synthesis, caspase activation, ROS production, LOX activation, quanylate cyclase activation, and finally, the influx of Ca^{2+} via a cGMP gated Ca^{2+} channel [30,40,41,43]. Flavonoids protect HT-22 cells from oxidative glutamate toxicity by interrupting the cell death cascade at three distinct steps; preventing the GSH decrease, blocking ROS production, and inhibiting Ca^{2+} influx (Table 2). Quercetin and fisetin increase GSH levels in HT-22 cells both in the presence and absence of glutamate (Fig. 5). Because glutamate decreases the intracellular level of GSH by inhibiting the uptake of cystine necessary for GSH production [33], the increase in GSH metabolism affords protection from glutamate. Such an increase in the basal level of GSH is often caused by the up-regulation of the rate-limiting enzyme for GSH metabolism, γ-GCS (Fig. 5B) [42,67]. A similar protective mechanism is observed with PG, MG (Fig. 5), and the agonist of metabotropic glutamate receptors DHPG [49]. It has been shown previously that the upstream region of the γ-GCS catalytic gene contains an electrophile responsive element [68] and that compounds such as β-naphthoflavone upregulate the regulatory subunit of γ-GCS [69].

Unlike the quercetin related compounds, which affect GSH metabolism, the galangin group protects HT-22 cells as antioxidants (Table 2). This conclusion is supported by five pieces of evidence. First, these compounds do not affect GSH levels (Fig. 5). Second, like vitamin E,

they maintain levels of glutamate-induced ROS within 2- to 10-fold of the control level (Fig. 6B; [59]). Third, these compounds are able to reduce levels of ROS that have been already accumulated in the cells (Fig. 7). Fourth, the TEAC values of these compounds indicate their antioxidative activities in a cell-free system (Table 1). Fifth, they also protect HT-22 cells from H_2O_2 (Fig. 3B). Because the galangin antioxidants can protect the cells despite the drop in intracellular GSH, GSH is acting as an endogenous antioxidant in the cell death cascade induced by glutamate [30,33].

The third mechanism of protection was observed with the flavonol type compounds (flavonol, 6-HF and 7-HF) (Table 2). These flavonols do not affect GSH metabolism (Fig. 5) nor do they act as antioxidants (Fig. 3B; Fig. 6B, Fig. 7; Table 1). Indeed, cells treated with the flavonols survive (Fig. 2) but accumulate ROS levels indistinguishable from those in cells dying from glutamate (Fig. 6B). Under normal conditions, the Ca^{2+} influx increases and cells die [30]. Despite the accumulation of high concentrations of ROS, the cells treated with the flavonols are able to survive with low levels of cytoplasmic Ca^{2+} (Fig. 8). The only protective compound that shows similar conditions of the metabolic markers is a nonspecific Ca^{2+} channel blocker Co^{2+} [30,38]. Co^{2+} blocks Ca^{2+} influx, resulting in the protection of the cells despite high ROS levels [30]. In a manner similar to Co^{2+}, these three flavonols may maintain low intracellular Ca^{2+} in spite of high levels of ROS. This conclusion is supported by the latent action of the flavonols in the time course experiment: the flavonols protect the cells even when added 10 h after glutamate (Fig. 4). The flavonols may interact directly with a Ca^{2+} channel that is responsible for the final demise of the cell [40], and prevent its opening. Alternatively, they may prevent the signaling mechanism between high ROS levels and the opening of the Ca^{2+} channel. We are currently investigating these possibilities.

It has not been formally ruled out that some of the flavonoids modify glutamate uptake, but this is not the mechanism used to protect the cells. For example, flavonol and galangin do not eliminate the loss of GSH caused by glutamate, showing that glutamate is still present in the medium. Quercetin and all other flavonoids are also protective in toxicity caused by BSO and cystine depletion, mechanisms independent of exogenous glutamate. Finally, the galangin group function directly as antioxidants.

As outlined above, individual flavonoids can protect HT-22 cells from oxidative stress via several different mechanisms (Table 2). Quercetin and fisetin, for example, alter GSH metabolism and act as antioxidants at the same time (Fig. 3B; Fig. 7; Table 1; data not shown). Baicalein and luteolin can act as lipoxygenase inhibitors [41], but they can also act as antioxidants (Table 1, Fig. 3B, Fig. 7). The latency of the efficacy of these flavonoids, as shown in Fig. 4, supports the importance of their action as antioxidants. Finally, some flavonoids may protect the cells from glutamate by directly inhibiting ROS production by mitochondria [61].

In summary, we have identified three protective mechanisms for flavonoids in a cell culture model of oxidative stress. The protective efficacy of flavonoids has also been shown in many animal models of oxidative stress. For example, red wine as well as constituent flavonoids such as quercetin reduce the progression of atherosclerosis in mice deficient in apolipoprotein E [70]. Because cellular oxidative stress is an important factor in various diseases, including arteriosclerosis, ischemia, trauma, Alzheimer's disease, Parkinson's disease, and AIDS as well as aging itself [27,71], flavonoids and flavonoid-containing foods may have multiple beneficial effects in the treatment of these conditions.

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ABBREVIATIONS

GSH—glutathione
ROS—reactive oxygen species
BSO—buthionine sulfoximine
H_2O_2—hydrogen peroxide
HCA—homocysteic acid
FBS—fetal bovine serum
DMEM—Dulbecco's modified Eagle's medium
MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS—phosphate-buffered saline
BSA—bovine serum albumin
SDS—sodium dodecyl sulfate
DCF—dichlorofluorescein
H_2DCF-dA—2′,7′-dichlorodihydrofluorescein diacetate
PI—propidium iodide
TEAC—Trolox equivalent activity concentration
ABTS—2,2′-azinobis(3-ehtylbenzothiazoline 6-sulfonate
6-HF—6-hydroxyflavonol
7-HF—7-hydroxyflavonol
γ-GCS—γ-glutamylcysteine synthetase
PG—propyl gallate
MC—methyl caffeate
DHPG—dihydrophenylglycine
FCCP—cyanide p-trifluoromethoxyphenylhydrazone
PBN—N-tert-butyl-α-phenylnitrone